

# PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 11 May 2000 (11.05.00)	
<b>International application No.</b> PCT/US99/20013	<b>Applicant's or agent's file reference</b> 1142.102-304
<b>International filing date (day/month/year)</b> 01 September 1999 (01.09.99)	<b>Priority date (day/month/year)</b> 01 September 1998 (01.09.98)
<b>Applicant</b> PAUSCH, Mark, Henry et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:  
30 March 2000 (30.03.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was  
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland	<b>Authorized officer</b> F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>1142.102-304</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/US 99/ 20013</b>	International filing date (day/month/year) <b>01/09/1999</b>	(Earliest) Priority Date (day/month/year) <b>01/09/1998</b>
Applicant <b>PAUSCH, Mark, Henry et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/20013

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7, 9, 13-14, 16-32, 34, 37 all partially

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: (1-7, 9, 13-14, 16-32, 34, 37) all partially

Nucleic acid encoding a rat M3 muscarinic acetylcholine receptor and polypeptide encoded therefrom comprising a deletion in the third intracellular loop of the GPCR. Nucleic acid encoding chimeric modified GPCR. Host cell. Method of screening.

2. Claims: (1-7, 9, 13-14, 16-32, 34, 37) all partially

Nucleic acid encoding a Drosophila melanogaster muscarinic acetylcholine receptor, polypeptide encoded therefrom comprising comprising a mutation/deletion in the third intracellular domain of the GPCR. Nucleic acid encoding chimeric modified GPCR. Host cell. Method of screening.

3. Claims: (1-7, 13-14, 16-32, 34, 37) all partially and 10 entirely

Nucleic acid encoding a rat cholecystokinin CCKB receptor, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a modified chimeric GPCR. Host cell. Method of screening.

4. Claims: (1-6, 13-14, 16-32, 34, 37) all partially and (11, 15) entirely

Nucleic acid encoding a rat somatostatin receptor subtype 3, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a chimeric receptor comprising a mutation in an intracellular domain of the receptor. Host cell. Method of screening.

5. Claims: (1-7, 13-14, 16-32, 34, 37) all partially and 12 entirely

Nucleic acid encoding a human alpha 2a adrenergic receptor, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a chimeric receptor comprising a mutation in an intracellular domain of the receptor. Host cell. Method of screening.

6. Claims: (1-6, 13-14, 16-32, 34, 37) all partially and 8 entirely

Nucleic acid encoding a C elegans serotonin (Ce 5HTR)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

receptor, polypeptide encoded therefrom comprising a mutation/deletion in the third intracellular domain. Nucleic acid encoding a modified chimeric GPCR. Host cell. Method of screening.

7. Claims: (27-31, 34, 37) all partially and (33, 35-36) all entirely

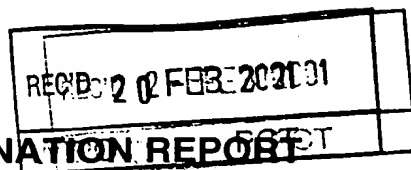
Host cell comprising a heterologous G-protein coupled receptor modified at the carboxy terminal tail. Method of screening using this host cell.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference 1142.102-304	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/20013	International filing date (day/month/year) 01/09/1999	Priority date (day/month/year) 01/09/1998
International Patent Classification (IPC) or national classification and IPC C12N15/12		
Applicant PAUSCH, Mark, Henry et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 9 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand  30/03/2000	Date of completion of this report  29.12.00
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Paresce, D  Telephone No. +49 89 2399 8995  

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/20013

## I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).):*

### Description, pages:

1-23 as originally filed

### Claims, No.:

1-12,14-17,20,24-29, with telefax of 14/12/2000  
32,33,35-38

### Drawings, sheets:

1/5-5/5 as originally filed

### Sequence listing part of the description, pages:

1-7, filed with the letter of 16.12.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/20013

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:  
**see separate sheet**

### III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 8, 10-12, 15, 33, 35-36 entirely, 1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 partially.

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 8, 10-12, 15, 33, 35-36 entirely, 1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 partially.

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

### V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/20013

## **citations and explanations supporting such statement**

### 1. Statement

Novelty (N)	Yes:	Claims	1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 (partially)
	No:	Claims	
Inventive step (IS)	Yes:	Claims	38
	No:	Claims	1-7, 9, 14, 16-17, 20, 24-29, 32, 37 (partially)
Industrial applicability (IA)	Yes:	Claims	1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 (partially)
	No:	Claims	

### 2. Citations and explanations **see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/20013

**Re Item I**

**Basis of the report**

This International Preliminary Examination Report is based on amended claims 1-12, 14-17, 20, 24-29, 32-33, 35-38 submitted with a telefax dated 14.12.00. The amended set of claims is supported by the original disclosure and therefore complies with the requirements of Article 34(2)(b) PCT.

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The Examining Division agrees with the objection put forward by the Search Division as to lack of unity (Rule 13.1 PCT). As the Applicant has not had a search report drawn up on the other invention (Rule 68.5 PCT), the application will be prosecuted on the basis of the invention in respect of which a search has already been carried out, in other words, the invention first mentioned in the claims. This includes the subject-matter of claims 1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 partially (see International Search Report). These claims are directed to nucleic acid encoding a **rat m3 muscarinic acetylcholine receptor** and the polypeptide encoded from said nucleic acid which nucleic acid comprises a deletion in the third intracellular loop of the GPCR (G protein-coupled receptor). The claims are further directed to nucleic acid encoding a chimeric modified GPCR, host cells and methods for screening compounds capable of binding to said GPCR.

Claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The Applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. The applicant should therefore, limit the application to the invention searched and excise those parts of the application relating to the other inventions.

**Re It m V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

- 1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.
- 2) D1 discloses the expression of heterologous GPCRs in yeast (in particular, *Saccharomyces cerevisiae*) and the use of said GPCRs in the development of high-throughput screening assays for GPCR ligands. D1 describes the various genetic techniques used to achieve heterologous GPCR expression in yeast (see p.488-490) and the development of sensitive assays that measure the functional response of said GPCRs (p.490-491). In particular, D1 describes the development of yeast cells functionally expressing the rat somastatin SST<sub>2</sub> receptor (coupled to the yeast pherome-response pathway) and the use of these yeast cells to screen for somastatin analogs and combinatorial libraries to identify novel subtype selective agonists and antagonists for said receptor. D1 further mentions that the described screening approaches, "can be implemented immediately for the increasingly large number of heterologous GPCRs that couple directly to the yeast G $\alpha$  protein" (p. 493). D1 also mentions the possibility of using heterologous GPCR expression systems in yeast to identify mutations that affect GPCR function, such as "improvement of heterologous GPCR interaction with the yeast G protein, induction of constitutive GPCR activity and the response to noncognate drugs" (p. 494).

D2 discloses that a serine and threonine rich domain regulates the internalization of muscarinic cholinergic receptors. Using deletion mutations, it was found that a small region in the middle of the third intracellular loop (i3) appears to be required for the agonist-induced internalization of the human m1 muscarinic cholinergic receptor. D2 describes studies using point mutations which were used to locate a specific domain (286-292) in the i3 loop of the human m1 muscarinic cholinergic receptor which is critical for internalization of the receptor. Mutations in analogous i3 domains also prevented receptor internalization in the human m3 muscarinic cholinergic receptor (Hm3) (see abstract). In particular, D2 discloses that a point mutation of the Hm3 sequence ENSASSD abolished agonist-induced

internalization whereas second messenger activation was unaffected as measured by PI turnover (p. 6864 and Table II). D2 also describes Hm3/Hm1 chimera receptors of which one, the Hm3/Hm1 loop (ALAAA) mutant was deficient in internalization (p. 6864).

D3 discloses that a tyrosine residue located at the beginning of the i3 domain of the rat m3 muscarinic receptor is critical for muscarinic receptor mediated stimulation of PI hydrolysis. Replacement of this residue with alanine or exchange of its position abolished receptor mediated PI hydrolysis in transfected COS-7 cells. Insertion of Tyr-254 into a functionally inactive chimeric m3/m2 muscarinic receptor yielded a mutant receptor that was able to stimulate PI hydrolysis to a similar maximum extent as the wild type m3 receptor in fact, the PI response induced by the mutant receptor was characterized by about 10-fold increased carbachol EC<sub>50</sub> value (see abstract as well as p. 11539, and Table II, figure 2B).

D4 discloses the expression in yeast of heterologous GPCRs, chimeric receptors comprising GPCR nucleotide sequences fused with sequences of G proteins and use thereof in bioassays (see abstract). D4 describes expression of the *Drosophila* muscarinic acetylcholine receptor (see p. 33) and of other receptors including SST2 in yeast (see p. 35-43). D4 further discloses that mutations in SST2 enhance the sensitivity of the responses to somatostatin (p.42-43).

D5 describes the identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. To study the role of a 16-17 amino acid segment at the N-terminal portion of i3 in conferring coupling selectivity, cDNAs encoding chimeric m2/m3 receptors were constructed (see introduction and figure 1). The ability of these receptor chimeras to stimulate PI hydrolysis was studied in transfected COS-7 cells (see results).

D7 describes modified G-protein coupled receptors having deletions in the third intracellular domain and methods of making said receptors. The modified receptors described in D7 bind agonists with high affinity in the presence or absence of the G-protein and can be used in high throughput radioligand binding assays to screen for high affinity ligands, regardless of whether the ligands are agonists or antagonists (see p. 2). The modified GPCRs are exemplified by the

hamster beta-2 adrenergic receptor, the human  $\beta 3$  receptor and the human 5HT-1D $\beta$  receptor. The examples describe the expression and cloning of said modified receptors into various expression vectors, including yeast expression vectors (example 12) as well as screening assays (see examples). Additionally D7 describes several other GPCRs that can be modified according to the methods described in the examples, one of the GPCRs mentioned is the muscarinic acetylcholine receptor (see p. 7).

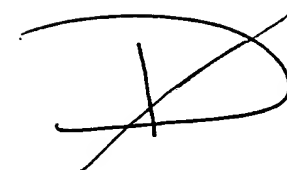
The subject-matter of claims 1-7, 9, 14, 16-17, 20, 24-29, 32, 37-38 (partially) has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel. The IPEA, however, is of the opinion that the subject-matter of claims 1-7, 9, 14, 16-17, 20, 24-29, 32, 37 partially does not meet the requirements of the PCT with respect to inventive step (Article 33(3) PCT).

At the priority date of the present application yeast cells functionally expressing heterologous GPCRs had been described. Many different GPCRs had been well characterized and the expression of heterologous GPCRs in yeast and use of heterologous GPCRs in the development of high-throughput screening assays had been described (for example D1 or D4). D1 describes the various genetic techniques used to achieve heterologous GPCR expression in yeast (see p.488-490) and the development of sensitive assays that measure the functional response of said GPCRs (p.490-491). D4 discloses the expression in yeast of heterologous GPCRs such as the *Drosophila* muscarinic acetylcholine receptor, chimeric receptors comprising GPCR nucleotide sequences fused with sequences of G proteins and use thereof in bioassays (see abstract).

Furthermore, several specific domains of the m3 muscarinic acetylcholine receptor had been identified in the prior art as being critical for the internalization or function of the receptor. D2 discloses that a serine and threonine rich domain regulates the internalization of muscarinic cholinergic receptors. Using deletion mutations, it was found that a small region in the middle of the third intracellular loop (i3) appears to be required for the agonist-induced internalization of the human m1 muscarinic cholinergic receptor. D3 discloses that a tyrosine residue located at the beginning of the i3 domain of the rat m3 muscarinic receptor is

critical for muscarinic receptor mediated stimulation of PI hydrolysis. D5 describes the identification of a 16-17 amino acid segment at the N-terminal portion of i3 of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. D7 describes modified G-protein coupled receptors having deletions in the third intracellular domain and methods of making said receptors. The modified receptors described in D7 bind agonists with high affinity in the presence or absence of the G-protein and can be used in high throughput radioligand binding assays to screen for high affinity ligands, regardless of whether the ligands are agonists or antagonists (see p. 2).

The amended set of claims are directed to yeast cells comprising a nucleic acid sequence encoding a modified rat m3 muscarinic acetylcholine receptor wherein the modification comprises a mutation in an intracellular domain of the receptor and which modification results in an improved functional response in a cell-based assay as compared to the wild-type form of the rat m3 muscarinic acetylcholine receptor. These yeast cells are developed using procedures that are slight modifications of procedures described in D7 or D4. These modifications are considered to come within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. D7 describes modified G-protein coupled receptors having deletions in the third intracellular domain and the expression of said modified receptors into yeast expression vectors (see above). It would be obvious to the person skilled in the art, namely when the same result is to be achieved, to apply these same features with corresponding effect to the rat m3 muscarinic acetylcholine receptor according to D2, D3, or D5. Furthermore, it is even mentioned in D7 that several other GPCRs can be modified according to the methods described in the examples, one of the GPCRs mentioned is the muscarinic acetylcholine receptor (see p. 7). The subject-matter of claims 1-7, 9, 14, 16-17, 20, 24-29, 32, 37 (partially) does not, therefore, involve an inventive step (Article 33(3) PCT).



We claim:

1. A yeast cell comprising a nucleic acid sequence encoding a modified, heterologous G protein-coupled receptor (GPCR), wherein the modification comprises a mutation in an intracellular domain of the G protein-coupled receptor and results in an improved functional response in a cell-based assay as compared to a wild-type form of the heterologous G protein-coupled receptor, and wherein the modified G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, a somatostatin receptor, an alpha 2A adrenergic receptor, and a serotonin receptor.
2. The yeast cell according to claim 1, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist.
3. The yeast cell according to claim 2, wherein the modification results in improved coupling between the receptor and a heterotrimeric G protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.
4. The yeast cell according to claim 1, wherein the mutation is a deletion.
5. The yeast cell according to claim 4, wherein the deletion is a point mutation.
6. The yeast cell according to claim 4, wherein the deletion is in the third intracellular loop of the G protein-coupled receptor.
7. The yeast cell according to claim 6, wherein the G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, and an alpha 2A adrenergic receptor.
8. The yeast cell according to claim 1, wherein the serotonin receptor is Ce 5HTR.
9. The yeast cell according to claim 1, wherein the muscarinic acetylcholine receptor is a rat M3 muscarinic acetylcholine receptor or a *D. melanogaster* muscarinic acetylcholine receptor.
10. The yeast cell according to claim 1, wherein the cholecystokinin CCKB receptor is a rat cholecystokinin CCKB receptor.
11. The yeast cell according to claim 1, wherein the somatostatin receptor is a rat somatostatin receptor subtype 3.

SUBSTITUTE PAGE

AMENDED SHEET

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12. The yeast cell according to claim 1, wherein the alpha 2A adrenergic receptor is a human alpha 2A adrenergic receptor.

14. The yeast cell according to claim 6, wherein the deleted third intracellular loop is 44 amino acids in length.

15. The yeast cell according to claim 11, wherein a sequence Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO:15) is deleted from the third intracellular loop of the mutant G protein-coupled receptor.

16. A yeast cell comprising a nucleic acid sequence encoding a chimeric G protein-coupled receptor, wherein the chimeric G protein-coupled receptor comprises a first heterologous G protein-coupled receptor in which an intracellular domain has been replaced with a modified intracellular domain of a second heterologous G protein-coupled receptor, and wherein the modified intracellular domain of the second heterologous G protein-coupled receptor confers an improved functional response to the chimeric G protein-coupled receptor in a cell-based assay as compared to a wild-type form of the first heterologous G protein-coupled receptor.

17. The yeast cell according to claim 16, wherein the modified intracellular domain is the third intracellular loop.

20. The yeast cell according to claim 1 or 16, further comprising a plasmid comprising an inducible reporter gene.

24. The yeast cell according to claim 20, wherein the reporter gene is a green fluorescent protein.

25. The yeast cell according to claim 24, wherein the green fluorescent protein is operably linked to a *FUS2* promoter.

26. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the yeast cell according to claim 1 or 16 to a test compound; and
- (b) measuring the effect of the test compound on cell growth.

27. A yeast cell comprising a heterologous G protein-coupled receptor, wherein the G protein-coupled receptor has a deletion in an intracellular domain that results in an improved functional response of the G protein-coupled receptor in a cell-based assay as compared to a wild-type form of the heterologous G protein-coupled receptor.

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AMENDED SHEET



28. The yeast cell according to claim 27, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist

29. The yeast cell according to claim 28, wherein the modification results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the heterologous G protein-coupled receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.

32. The yeast cell according to claim 27, wherein the intracellular domain is the third intracellular domain.

33. The yeast cell according to claim 27, wherein the heterologous G protein-coupled receptor is modified at the carboxy terminal tail of the G protein-coupled receptor.

35. The yeast cell according to claim 33, wherein the modified G protein-coupled receptor is a neurotensin receptor.

36. The yeast cell according to claim 35, wherein the neurotensin receptor is a rat neurotensin NT1 receptor.

37. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the yeast cell according to claim 27 to a test compound; and
- (b) measuring the effect of the test compound on yeast cell growth.

38. The yeast cell according to claim 6, wherein the deletion is IC3A.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 99/20013

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/705 C07K14/72 C12N15/62 C12Q1/02  
C12N1/19 C12N15/63 C12N15/67

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PAUSCH M H: "G-protein-coupled receptors in Saccharomyces cerevisiae: high-throughput screening assays for drug discovery"</p> <p>TRENDS IN BIOTECHNOLOGY, GB, ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 15, no. 12, 1 December 1997 (1997-12-01), pages 487-494, XP004097426</p> <p>ISSN: 0167-7799</p> <p>cited in the application</p> <p>the whole document especially page 494</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/-</p>	<p>1-7,9, 16-32, 34,37</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

20 April 2000

Date of mailing of the international search report

31.07.00

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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/20013

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>O. MORO ET AL: "Serine-and Threonine-rich Domain regulates internalization of muscarinic cholinergic Receptors" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 268, no. 10, 5 April 1993 (1993-04-05), pages 6862-6865, XP002135333 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 cited in the application</p>	1-7,13, 16-21, 27,30-32
A	<p>the whole document especially page 6864 left column, table II</p>	37
X	<p>---            BLUEML K ET AL: "FUNCTIONAL ROLE OF A CYTOPLASMIC AROMATIC AMINO ACID IN MUSCARINIC RECEPTOR-MEDIATED ACTIVATION OF PHOSPHOLIPASE C" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 269, no. 15, 15 April 1994 (1994-04-15), pages 11537-11541, XP002911766 ISSN: 0021-9258 the whole document</p>	1,4-7,9
A	<p>---            WO 95 21925 A (AMERICAN CYANAMID CO ;PAUSCH MARK HENRY (US); OZENBERGER BRADLEY A) 17 August 1995 (1995-08-17) page 31; claims; example 14</p>	1-7,9, 16-32, 34,37
X	<p>---            J. WESS ET AL: "Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover" FEBS LETTERS., vol. 258, no. 1, November 1989 (1989-11), pages 133-136, XP002135334 AMSTERDAM NL the whole document</p>	1,4-7,9, 16-19, 27,30-32
A	<p>---            WESS J: "G protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition" FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 11, no. 5, April 1997 (1997-04), pages 346-354, XP002121229 ISSN: 0892-6638 figures 1,2</p>	1-7,9, 13,14, 16-32, 34,37
	<p>---            -/--</p>	

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/20013

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 00739 A (MCALLISTER GEORGE ;STRADER CATHERINE D (US); DIXON RICHARD (US); G) 11 January 1996 (1996-01-11) the whole document especially page 2, line 26 - line 30	27-32,37
A	page 1 -page 3; claims; example 12 page 4, line 11 -page 5, line 3 page 12, line 25 -page 13, line 13 claims; examples 12,15	1-6,13
T	--- PAUSCH M H ET AL: "HETEROLOGOUS G PROTEIN-COUPLED RECEPTORS EXPRESSED IN SACCHAROMYCES CEREVISIAE: METHODS FOR GENETIC ANALYSIS AND LIGAND IDENTIFICATION" IDENTIFICATION AND EXPRESSION OF G-PROTEIN-COUPLED RECEPTORS, 26 May 1999 (1999-05-26), pages 196-212, XP000869632 Edited by Kevin R. Lynch ISBN 0-471-19493-X. Wiley Liss, INC. cited in the application	
T	--- SCHEER A ET AL: "CONSTITUTIVELY ACTIVE G PROTEIN-COUPLED RECEPTORS: POTENTIAL MECHANISMS OF RECEPTOR ACTIVATION" JOURNAL OF RECEPTOR AND SIGNAL TRANSDUCTION RESEARCH,US,MARCEL DEKKER, NEW YORK, NY, vol. 17, no. 1/03, 1997, pages 57-73, XP000867531 ISSN: 1079-9893 -----	

# INTERNATIONAL SEARCH REPORT

tional application No.  
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## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
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3. ☐ Claims Nos.:  
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## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7, 9, 13-14, 16-32, 34, 37 all partially

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/20013

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: (1-7, 9, 13-14, 16-32, 34, 37) all partially

Nucleic acid encoding a rat M3 muscarinic acetylcholine receptor and polypeptide encoded therefrom comprising a deletion in the third intracellular loop of the GPCR. Nucleic acid encoding chimeric modified GPCR. Host cell. Method of screening.

2. Claims: (1-7, 9, 13-14, 16-32, 34, 37) all partially

Nucleic acid encoding a Drosophila melanogaster muscarinic acetylcholine receptor, polypeptide encoded therefrom comprising comprising a mutation/deletion in the third intracellular domain of the GPCR. Nucleic acid encoding chimeric modified GPCR. Host cell. Method of screening.

3. Claims: (1-7, 13-14, 16-32, 34, 37) all partially and 10 entirely

Nucleic acid encoding a rat cholecystokinin CCKB receptor, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a modified chimeric GPCR. Host cell. Method of screening.

4. Claims: (1-6, 13-14, 16-32, 34, 37) all partially and (11, 15) entirely

Nucleic acid encoding a rat somatostatin receptor subtype 3, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a chimeric receptor comprising a mutation in an intracellular domain of the receptor. Host cell. Method of screening.

5. Claims: (1-7, 13-14, 16-32, 34, 37) all partially and 12 entirely

Nucleic acid encoding a human alpha 2a adrenergic receptor, polypeptide encoded therefrom comprising a deletion in the third intracellular loop. Nucleic acid encoding a chimeric receptor comprising a mutation in an intracellular domain of the receptor. Host cell. Method of screening.

6. Claims: (1-6, 13-14, 16-32, 34, 37) all partially and 8 entirely

Nucleic acid encoding a C elegans serotonin (Ce 5HTR)

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/20013

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

receptor, polypeptide encoded therefrom comprising a mutation/deletion in the third intracellular domain. Nucleic acid encoding a modified chimeric GPCR. Host cell. Method of screening.

7. Claims: (27-31, 34, 37) all partially and (33, 35-36) all entirely

Host cell comprising a heterologous G-protein coupled receptor modified at the carboxy terminal tail. Method of screening using this host cell.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/20013

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9521925 A	17-08-1995	US 5691188 A	25-11-1997
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/12, C07K 14/705, 14/72, C12N 15/62, C12Q 1/02, C12N 1/19, 15/63, 15/67</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/12705</b> <b>(43) International Publication Date:</b> 9 March 2000 (09.03.00)
<b>(21) International Application Number:</b> PCT/US99/20013 <b>(22) International Filing Date:</b> 1 September 1999 (01.09.99) <b>(30) Priority Data:</b> 60/098,704 1 September 1998 (01.09.98) US <b>(71)(72) Applicants and Inventors:</b> PAUSCH, Mark, Henry [US/US]; American Cyanamid Company, P.O. Box 400, Clarksville, NJ 08543-0400 (US). WESS, Jurgen [-/US]; Laboratory of bioorganic Chemistry, NIH-NIDDK, Building 8A, Room B1A-05, 9000 Rockville Pike, Bethesda, MD 20892 (US). <b>(74) Agents:</b> GARRETT, Arthur, S. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street, N.W., Washington, DC 20005-3315 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> METHODS FOR IMPROVING THE FUNCTION OF HETEROLOGOUS G PROTEIN-COUPLED RECEPTORS  <b>(57) Abstract</b>  This invention relates to mutant G protein-coupled receptors with improved G-protein coupling and receptor response, yeast cells expressing such receptors, vectors useful for making such cells, and methods of making and using same.		

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## METHODS FOR IMPROVING THE FUNCTION OF HETERLOGOUS G PROTEIN-COUPLED RECEPTORS

### STATEMENT OF RELATED APPLICATIONS

This application hereby claims the benefit of United States provisional application Serial No. 60/098,704 filed September 1, 1998. The entire disclosure of this provisional application is relied upon and incorporated by reference herein.

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### FIELD OF THE INVENTION

This invention relates to mutant G protein-coupled receptors with improved G-protein coupling and receptor response, host cells expressing such receptors, vectors useful for making such cells, and methods of making and using same.

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### BACKGROUND OF THE INVENTION

The actions of many extracellular signals, such as neurotransmitters, hormones, odorants, and light, are mediated by a triad of proteins which has been identified in organisms from yeast to mammals. This triad consists of a receptor, coupled to a trimeric guanine nucleotide-binding regulatory protein (G protein), which in turn is coupled to a cellular effector. These receptors have seven transmembrane domains and are named for their association with the G protein as "G protein-coupled receptors" ("GPCRs").

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The regulatory G proteins are comprised of three subunits: a guanylnucleotide binding  $\alpha$  subunit; a  $\beta$  subunit; and a  $\gamma$  subunit. *B.R. Conklin and H.R. Bourne (1993)*. G proteins cycle between two forms, depending on whether GDP or GTP is bound to the  $\alpha$  subunit. When GDP is bound, the G protein exists as a heterotrimer, the  $G\alpha\beta\gamma$  complex. When GTP is bound, the  $\alpha$  subunit dissociates, leaving a  $G\beta\gamma$  complex. Importantly, when a  $G\alpha\beta\gamma$  complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of disassociation of the bound  $G\alpha$  subunit from the  $G\beta\gamma$  complex increases. The free  $G\alpha$  subunit and  $G\beta\gamma$  complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways. Examples of these downstream cellular effector proteins include, among others, adenylate cyclases, ion channels, and phospholipases. This

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fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. *H.G. Dohlman et al. (1991).*

Because of their ubiquitous nature in important biochemical pathways, the G protein-coupled receptors represent important targets for new therapeutic drugs. In turn, the discovery of such drugs will necessarily require screening assays of high specificity and throughput termed high-throughput screening (HTS) assays. Screening assays utilizing microorganisms, such as yeast strains, genetically modified to accommodate functional expression of the G protein-coupled receptors offer significant advantages in research involving ligand binding to numerous receptors implicated in various disease states.

However, microorganisms transformed with wild-type receptors may perform poorly in growth assays, exhibiting, for example, the inability to interact with the heterotrimeric G protein, inappropriate localization and/or desensitization. Many GPCRs are phosphorylated in response to chronic and persistent agonist stimulation which often leads to desensitization followed by sequestration or internalization of the receptors. Desensitization of GPCRs causes uncoupling from interaction with heterotrimeric G proteins. This process is mediated by a variety of regulatory receptor protein kinases, including G protein-coupled receptor kinases (GRK), protein kinase A (PKA), protein kinase C (PKC), and casein kinases (CK). Internalization involves removal of GPCRs from the plasma membrane via receptor-mediated endocytosis. Internalized receptors may be recycled back to the cell surface, or delivered to a lysosomal/vacuolar compartment for degradation. The ubiquitin-mediated degradative pathway is also involved in this process. The ultimate result of receptor phosphorylation and sequestration/internalization is often cell growth arrest, which significantly reduces the utility of the genetically modified microorganism in screening assays.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide modified G protein-coupled receptors that function well in high throughput screening assays, implemented in any eukaryotic cell, preferably yeast cells. Thus, a first aspect of the present invention is

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directed to nucleotide sequences encoding a G protein-coupled receptor which has been modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery, and/or to appropriately localize to the plasma membrane. In preferred embodiments, such modifications lead to improved agonist-stimulated growth-promoting ability. One specific modification of the nucleotide sequence encoding a G protein-coupled receptor encompassed by this invention is a mutation in any intracellular domain or membrane region proximal to internal domains. The mutation may be a deletion, including, for example, a point mutation.

This invention is also directed to chimeric GPCRs in which intracellular domains of heterologous GPCRs that provide favorable G protein coupling properties or domains not subject to the yeast cell desensitization and/or sequestration/internalization machinery are used to replace comparable domains in GPCRs of interest. This invention also relates to the modified nucleotide sequences encoding the chimeric GPCRs, to expression vectors comprising the modified nucleotide sequences, and to host cells transformed therewith.

An additional aspect of this invention is an improved method of assaying compounds to determine effects of ligand binding to the mutant or chimeric GPCRs of this invention by measuring the effect of the test compound on cell growth. The mutant GPCRs prevent or reduce the rate of cell growth arrest due to chronic and persistent agonist stimulation, thereby decreasing the number of false negatives that occur with prior art screening methods and/or increasing the sensitivity of the bioassay.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**FIGURE 1A** depicts the results of liquid culture assays on yeast cells containing the rat M3 muscarinic acetylcholine receptor (MAR) using MAR agonist carbachol (CCh). Yeast cells containing a M3 MAR with a deletion in the third intracellular loop (IC3Δ) produced an agonist-dependent growth response, while the wild type MAR did not, indicating that the M3 MAR IC3Δ is functional GPCR.

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**FIGURE 1B** depicts the results of liquid culture fluorescence induction assays on yeast cells containing the rat M3 MAR IC3Δ and the FUS2-GFP reporter plasmid using the MAR agonist carbachol (CCh). A dose-dependent increase in the expression of the green fluorescent protein is observed in response to CCh activation of the M3 MAR IC3Δ expressed in yeast.

**FIGURE 2** depicts the results of liquid culture assays on yeast cells containing the *Drosophila* muscarinic acetylcholine receptor using the MAR agonist carbachol (CCh). Yeast cells containing a mutated *Drosophila* MAR containing the M3 MAR IC3Δ produced an agonist-dependent growth response while the wild type *Drosophila* MAR lacked an agonist-dependent yeast cell growth response.

**FIGURE 3** depicts the results of an agar-based plate bioassay. **FIGURE 3A** shows a robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor. **FIGURE 3B** shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of a portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

**FIGURE 4** depicts yeast cells transformed with rSSTR3 and with rSSTR3ΔIC3. **FIGURE 4A** demonstrates that yeast cells containing p426GPD-rSSTR3 show a weak response to somatostatin (S-14). **FIGURE 4B** demonstrates a much stronger response by yeast cells containing p426GPD-rSSTR3ΔIC3 assayed under similar conditions.

**FIGURE 5** depicts the results of liquid culture assays on yeast cells containing wild type IC3Δ human alpha2A adrenergic receptor using the alpha adrenergic receptor full agonist UK14304. Yeast cells containing the wild type and IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that the IC3 deletion is functional.

**FIGURE 6** depicts the results of liquid culture assays on yeast cells containing wildtype and carboxy terminally truncated rat NT1-neurotensin receptors using the neurotensin receptor agonist AcNT8-13. Truncation of the rat NT1-neurotensin receptor produces an agonist-dependent growth response that is more sensitive than that observed with the wildtype receptor.

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**FIGURE 7** depicts the results of liquid culture assays on yeast cells containing the *C. elegans* serotonin receptor using serotonin (5HT) to stimulate yeast cells growth. Yeast cells containing a mutated *C. elegans* serotonin receptor containing the M3 MAR IC3Δ produced a 5HT-dependent growth response. The growth response was blocked by addition of the serotonin receptor antagonists lisuride and mianserin.

### DETAILED DESCRIPTION OF THE INVENTION

#### Modified G Protein-Coupled Receptors

Nucleotide sequences encoding G protein-coupled receptors may be modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery. Such modifications lead to improved agonist-stimulated yeast cell growth-promoting ability. The improvement of GPCR-G protein coupling and/or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell provides a means to improve the function of wildtype heterologous GPCRs that fail to stimulate a useful yeast cell growth response. Thus, GPCRs that fail to function in their wild type form may be made to work by the methods of this invention.

The improvement of GPCR-G protein coupling and or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell may be assessed by using routine techniques, such as those described in the Examples set forth below or known to those of skill in the art. For example, improvement of the function of a mutated GPCR over wild type may be quantified as an increase in the signal-to-noise ratio and/or in the sensitivity of the liquid bioassay. The signal-to-noise ratio is determined by comparing the agonist-induced growth rate to the growth rate observed in the absence of agonist. A statistically-significant increase in the signal-to-noise ratio resulting from agonist-stimulation of a mutated GPCR over similar values obtained from cells containing a wild type receptor indicates that the function of the mutated GPCR has been improved.



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The sensitivity of the liquid bioassay is defined as the agonist concentration necessary to produce a half-maximal growth rate (ED50 or EC50). The sensitivity of the bioassay is increased if a mutated GPCR produces a half-maximal growth rate at an agonist concentration that is less than that required by the wild type GPCR.

5 Similarly, the more qualitative agar based bioassay will reflect increases in signal-to-noise ratio and/or sensitivity due to agonist stimulation of mutated GPCRs. In the agar based bioassay, signal-to-noise ratio increases are determined by comparing the extent of growth within the agonist induced growth zone resulting from stimulation of mutated and wild type receptor. The sensitivity of the bioassay is  
10 proportional to the radius of the growth zone. Since applied compounds diffuse radially from the site of application to the agar, agonist concentration varies with the square of the radius of the growth zone. Thus, a larger zone of growth in response to agonist activation of mutated GPCRs reflects an increase in sensitivity.

Any G protein-coupled receptor may be employed in practicing this invention.  
15 Examples of such receptors include, but are not limited to, adenosine receptors, somatostatin receptors, dopamine receptors, cholecystokinin receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, serotonin receptors, vasopressin receptors, melanocortin receptors, and neurotensin receptors.  
20 In certain preferred embodiments, the receptor is a muscarinic acetylcholine receptor and more preferably, the muscarinic acetylcholine receptor is of the M3 subtype.

Similarly, any suitable host cell may be transformed with the nucleotide sequences encoding the modified G protein-coupled receptors of this invention. Examples of suitable host cells are yeast cells, mammalian cells, insect cells, and  
25 bacterial cells. Preferably, the host cells are yeast cells.

One generalizable method for improving the function of a GPCR expressed in a host cell is by modification or elimination of intracellular domains of the GPCR, such as the third intracellular loop (IC3) sequences of the G protein-coupled receptor. Because the desensitization and internalization machinery acts upon the intracellular  
30 domains of the GPCR, elimination of the intracellular domains of the GPCRs produces a more stable receptor expression. This has been demonstrated in

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experiments conducted in mammalian cells. Muscarinic acetylcholine receptors, including the M3 subtype, lacking a domain of their third intracellular loop thought to be involved in receptor internalization, are maintained in the plasma membrane to a greater extent than their wild type counterparts. *Moro et al. (1993)*.

5           Representative embodiments of the invention are described in more detail in the following examples.

**Example 1. Functional Expression of A Mutated Rat M3 Muscarinic Acetylcholine Receptor (MAR) in Yeast**

10           The third intracellular loops of GPCRs are thought to interact with and participate in the activation of G proteins upon agonist binding. *J. Wess (1997)*. Mutations in IC3 of the yeast mating pheromone receptors, Ste2 and Ste3 have profound effects on coupling the G proteins. *C. Boone et al. (1993)* and *C. Clark et al. (1994)*. Importantly, deletion of portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in  
15           mammalian cells with retention of full ability to couple to the heterotrimeric G protein, Gq ( $G\alpha\beta\gamma$ ). The mutated M3 MAR retains all external loops. Transmembrane domains (TMDs) and internal domains other than the IC3 are unchanged. The IC3, found between 5th and 6th membrane spanning helices, was the  
20           only domain modified. The bulk of this domain, 96 amino acids in the center of the IC3 (Ala273-Lys469), were deleted, leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, the third intracellular loop of the MAR containing the IC3 deletion (IC3 $\Delta$ ) is 44 amino acids in length, compared to 240  
25           amino acids in the IC3 of wild type M3 MAR. The improvement in functional expression may due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

30           In order to test the possibility that this IC3 $\Delta$  mutation would also improve functional expression in yeast, the DNA sequences encoding the wild type and IC3 $\Delta$  rat M3 MARs were cloned into proximity to the glycerol-phosphate dehydrogenase promoter in the yeast expression plasmid, p426GPD, by standard methods. Rat M3

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MAR sequences were amplified by PCR using oligonucleotides containing 5' *Bgl*III (AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *Xho*I (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. The resulting PCR product was digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. To form the rat M3 IC3Δ, three M3 MAR fragments were amplified by PCR. An amino-terminal coding fragment was amplified using oligonucleotides containing 5' *Bgl*III (AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *Age*I (ATAGTCATGATGGTG ACCGGT ATGTAAAAGGCAGCGATC) (SEQ ID NO: 3) sites. A carboxy-terminal coding fragment was amplified using oligonucleotides containing 5' *Pml*I (GCCTTCATCAT CACGTG GACCCCCTACACC) (SEQ ID NO: 4) and 3' *Xho*I (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. An IC3 coding fragment was amplified using oligonucleotides containing 5' *Age*I (CGATCGCTGCCTTTTACTT ACCGGT CACCATCATGACTAT) (SEQ ID NO: 5) and 3' *Pml*I (GTTGTAGGGGGTGC CACGTG ATGATGAAGGC) (SEQ ID NO: 6) sites using the M3 IC3Δ sequence. *J. Wess (1997)*. The resulting PCR products were digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. Plasmids were confirmed by restriction endonuclease mapping and DNA sequencing. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, incorporated herein by reference, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

Yeast cells containing the MARs were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples ( $10^{-1}$ - $10^{-8}$  M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600

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rpm). Growth was monitored by recording increases in OD<sub>620</sub> using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean  $\pm$  SEM and were plotted vs. agonist concentration. As shown in Figure 1, the yeast cells containing the M3 MAR IC3 $\Delta$  produced an agonist-dependent growth response, demonstrating that the M3 MAR IC3 $\Delta$  is functional, while the wild type MAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth. The growth response of the M3 MAR IC3 $\Delta$  containing cells was dose-dependent giving an EC<sub>50</sub> for carbachol (CCh) equal to 3  $\mu$ M. This value matches the K<sub>D</sub> for CCh obtained in HEK cells (7.9  $\mu$ M) and the EC<sub>50</sub> for CCh induced IP<sub>3</sub> (inositol triphosphate) accumulation (4.0  $\mu$ M), suggesting that the M3 MAR IC3 $\Delta$  retains the expected pharmacological properties when expressed in yeast cell membranes. Further, the growth response is blocked by the MAR-specific antagonist, atropine (At).

Alternatively, the response to CCh by yeast cells containing the M3 MAR IC3 $\Delta$  may be observed by measuring the agonist-induced increase in fluorescent emission from a green fluorescent protein reporter gene whose expression is stimulated by MAR agonists. Green fluorescent protein (GFP) is a protein from *Aequorea* that is intrinsically fluorescent when expressed in yeast cells. The fluorescence from GFP is detectable in live yeast cells, making it possible to measure the level of its expression without any deleterious treatment of the yeast cells. This feature is particularly advantageous in the reporter gene assays that do not require additional steps to permit its detection. An inducible reporter gene that is useful in detecting the agonist-activation of heterologous GPCRs expressed in yeast utilizes transcriptional promoters that are activated by the mating signal transduction pathway. One such promoter is the *FUS2* promoter. In the absence of agonist stimulation, little or no expression of the Fus2 protein or any other protein whose expression is directed by the *FUS2* promoter is detectable. After treatment with agonist, transcription from the *FUS2* promoter is induced up to 700 fold, leading to substantial increases in Fus2 expression or in the expression of any gene product whose expression is placed under control of the *FUS2* promoter. Thus, yeast cell fluorescence resulting from GFP

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expression under the control of the *FUS2* promoter from a *FUS2*-GFP reporter gene is only observed after agonist activation of a heterologous GPCR.

In order to produce a GFP reporter gene, DNA sequences encoding the enhanced GFP (EGFP, Clontech), *FUS2* promoter and *FUS2* transcriptional terminator sequences were amplified by PCR. The fragments were assembled into the centromere containing plasmid pRS414 so as to place EGFP expression under control of the pheromone responsive *FUS2* promoter in the centromere containing plasmid pRS414, producing plasmid pMP241. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188, that are useful for performing assays of GPCR agonist-stimulated growth of cells containing the M3 MAR IC3 $\Delta$ . Specifically, the plasmids were introduced into the MPY578fc cells described in Pausch *et al.* (1998).

Yeast cells containing the M3 MAR IC3 $\Delta$  and the *FUS2*-EGFP reporter plasmid were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were washed and diluted 5 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180  $\mu$ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20  $\mu$ l of serially-diluted samples ( $10^{-1}$ - $10^{-8}$  M) of CCh. The plates were incubated at 30°C for 6 hours with agitation (600 rpm). Stimulation of the *FUS2*-EGFP reporter gene expression was monitored by recording increases in emission at 530 nm after excitation with 480 nm light using a fluorescence microplate reader. Assays were conducted in duplicate and measurements obtained during the logarithmic phase of yeast cell growth. Fluorescence emission measurements were analyzed using GraphPad Prism and were presented as the mean  $\pm$  SEM and were plotted vs. agonist concentration. As shown in Figure 1B, the yeast cells containing the M3 MAR IC3 $\Delta$  produced a dose dependent increase in fluorescence emission, consistent with increased expression of the EGFP from the agonist inducible *FUS2*-GFP reporter gene construct. The EC50 for CCh stimulation of fluorescence emission is 4  $\mu$ M, identical to values obtained in the growth assay.

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Thus, deletion of a portion of the IC3 of the rat M3 MAR has produced a functional GPCR when expressed in yeast, suggesting that modification of internal domains may be a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

**Example 2. Functional Expression of a Mutated *D. melanogaster* Muscarinic Acetylcholine Receptor in Yeast**

Agonist of the G protein-coupled insect muscarinic acetylcholine receptors (MARs) possess substantial insecticidal and miticidal activity. *M.R. Dick et al. (1997)*. These observations suggest that development of a yeast-based high throughput screen (HTS) for agonists active at insect MARs may be useful in identifying lead compounds that might be developed into insecticides with novel mode of action. Preliminary experiments indicate that the wild type *D. melanogaster* MAR (DMAR), an insect G protein-coupled receptors (GPCRs), is non-functional in yeast. Thus, an effort to develop a method for improving the function of the DMAR in yeast was mounted, via replacement of the DMAR IC3 with the functional IC3 from the M3 MAR IC3Δ.

In insect cells, the DMAR interacts with the heterotrimeric Gq protein leading to an increase in intracellular calcium in response to muscarinic agonists. One potential explanation for the inactivity of the DMAR in yeast is an inability to efficiently couple to the yeast heterotrimeric G protein. Thus, to devise a method to improve the DMAR function in yeast, selected mutations in the GPCRs that serve to improve functional expression and coupling to the heterotrimeric G protein were examined.

In order to construct the IC3 replacement, PCR fragments encoding three domains were prepared by standard means. Fragment 1 consisted of the amino terminal coding portion of the *Drosophila* MAR up to an *AgeI* site within the 5th TMD, amplified by PCR using oligonucleotides (AAA AGATCT AAA ATG TACGGAAACCAGACGAAC) (SEQ ID NO: 7) and (CCA GTA GAG GAA GCACATGATGGTC AGGCCT AAG TAG AAG GCG GCC AGT GC) (SEQ ID NO: 8). The second fragment of the DMAR was composed of carboxy terminal

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coding sequences starting with a *PmII* site in the 6th TMD, amplified by PCR using oligonucleotides (TTCATCATCACGTGGACTCCGTACAACATC) (SEQ ID NO: 9) and (AAA CTCGAG TTATCTAATTGTAGACGCGGC) (SEQ ID NO: 10). The M3 MAR IC3 $\Delta$  domain was amplified as an *AgeI*-*PmII* fragment with coding sequence in frame with fragments 1 and 2, described in Example 1. These fragments were assembled in plasmid p426GPD to place the mutated DMAR under control of the GPD promoter. The wild type DMAR was cloned into the expression vector, pMP3, described in United States Patent 5,691,188. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

Yeast cells containing the DMAR and the plasmid containing the wild type DMAR were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8, medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180  $\mu$ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20  $\mu$ l of serially-diluted samples ( $10^{-1}$  -  $10^{-8}$  M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean  $\pm$  SEM and were plotted vs. agonist concentration. As shown in Figure 2, the yeast cells containing the mutated DMAR, i.e., the M3 MAR IC3 $\Delta$ , produced an agonist-dependent growth response, demonstrating that the DMAR-M3 MAR IC3 $\Delta$  is functional. The wild type DMAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth.

Thus, replacing IC3 of the DMAR with the functional deleted IC3 from the rat M3 MAR produces a functional chimeric GPCR when expressed in yeast, indicating that this method of replacing internal domains may be a generalizable method for

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improving the functional expression of heterologous GPCRs in cell-based assays, such as yeast assays.

**Example 3. Functional Expression of a Mutated Rat Cholecystokinin CCKB Receptor in Yeast**

As shown in Examples 1 and 2, deletion of portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. In order to test the possibility that this IC3 $\Delta$  mutation would also improve functional expression of other GPCRs in yeast, the DNA sequences encoding the rat wild type and IC3 $\Delta$  cholecystokinin CCKB receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The wild type CCKBR was amplified by PCR using oligonucleotides (ACTTAGATCAAAAAATGGAGCGCTCAAGCTGAACCG) (SEQ ID NO: 11) and (TCCCGTCGACTCAGCCAGGCCCCAGTGTGCTG) (SEQ ID NO: 12). The IC3 $\Delta$  cholecystokinin CCKB receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 22 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides (ACTTAGATCAAAAAATGGAGCGCTCAAGCTGAACCG) (SEQ ID NO: 11) and (CGAGGGCCAGGGACTGGCCCCGGCCGGGCGGCTTTGGGTCTCG) (SEQ ID NO: 13). Fragment 2 contained carboxy terminal coding sequences including 22 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides (TCCCGTCGACTCAGCCAGGCCCCAGTGTGCTG) (SEQ ID NO: 12) and (CGAGACCCAAAGCCGGGCCCCGGCCGGGCGGAGTCCCTGGCCCTCG) (SEQ ID NO: 14). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length CCKB receptor. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.



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Yeast strains containing wild type and IC3Δ cholecystokinin CCKB receptor were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil (SCD-ura) medium. In this agar-based plate bioassay, molten (50°C) SCD-ura-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH<sub>4</sub>OH prior to autoclaving) containing 0.5 mM AT (3-aminotriazole) was inoculated with the overnight culture (2 x 10<sup>4</sup> cells/ml) and poured into square (9 x 9 cm) petri plates. Solutions of CCK agonists in DMSO (1 mM, 10 μl) were applied to the surface of the solidified agar (Upper left: CCK8S; upper right, CCK8US; lower left, CCK5; lower right, CCK4). Compounds applied to the surface of the plate diffused radially from the site of application and bound to CCKB receptors expressed on the surface of cells embedded in the agar, resulting in induction of *FUS1-HIS3* expression. The responding cells formed a dense growth zone readily detectable over the limited growth of cells observed in response to basal *FUS1-HIS3* expression. Plates were incubated at 30°C for 3 days (Figure 3). Figure 3A demonstrates the robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor, while Figure 3B shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

#### **Example 4. Functional Expression of a Mutated Rat Somatostatin Receptor (SSTR) in Yeast**

The third intracellular loop participates in many GPCR functions, including G protein coupling, desensitization and interaction with diverse modifying proteins. Somatostatin receptors are encoded in five subtypes, labeled SSTR1-5. Several amino acids are found in the third intracellular loop of the SSTR3 subtype, but not in the equivalent region of SSTR2 subtype. Since SSTR2 functions efficiently in yeast, deletion of those amino acids from IC3 may impart this functional efficiency upon SSTR3. Thus, 8 amino acids, Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO: 15), were deleted from the third intracellular loop of the rSSTR3 cDNA, enabling more efficient receptor signaling in yeast.

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Rat SSTR3 sequences were amplified by PCR using oligonucleotides containing 5' BglII and 3' XhoI sites. The resulting PCR product of approximately 1.3 kb was digested with BglII and XhoI, purified and inserted between the BamHI and XhoI sites in p426GPD to generate the plasmid p426GPD-rSSTR3. Recombinant plasmids were confirmed by restriction endonuclease digestion and DNA sequencing.

Standard PCR reactions were used to amplify the rSSTR3 cDNA to yield two PCR fragments that have 36 bp overlap as follows. PCR insert A of approximate size 750 bp was generated using the 5' Bgl oligonucleotide (AAAAAGATCT AAAATGGCCA CTGTTACCTA T) (SEQ ID NO: 16) and the 3' oligonucleotide CTCAGAGCGG CGTCGCCGCT GACACGAGGG CGCCCG (SEQ ID NO: 17). PCR insert B of approximate size 530 bp was generated using the 5' oligonucleotide GCGCCCTCGT GTCAGCGGCG ACGCCGCTCT GAG (SEQ ID NO: 18) and the 3' XhoI oligonucleotide (AAAACTCGAG TTACAGATGG CTCAGTGTGC T) (SEQ ID NO: 19). PCR fragments A and B were gel purified, annealed and amplified by PCR using the flanking 5' BglII and 3' XhoI oligonucleotides to yield the ~1.3 kb rSSTR3 $\Delta$ IC3 PCR product. Following purification and digestion with BglII-XhoI, the rSSTR3 $\Delta$ IC3 insert was ligated into BamHI-XhoI sites of p426GPD to generate the plasmid p426GPD-rSSTR3 $\Delta$ IC3. Restriction mapping and DNA sequencing confirmed correct reading frame and sequence.

Yeast cells of the type useful for expression of GPCRs, described in United States Patent 5,691,188, were transformed with p426GPD-rSSTR3 and p426GPD-rSSTR3 $\Delta$ IC3, using standard procedures. The cells (i.e., the LY296 cells, described in *Price et al. (1995)*) were assayed using the agar-based bioassay format described in Example 3. Samples (10  $\mu$ l) of Somatostatin (S-14, 1mM) were applied to the surface of the selective agar medium containing the yeast cells expressing the SSTR3. The plates were incubated for 3 days at 30°C. Yeast cells transformed with p426GPD-rSSTR3 along with pLP82 (containing a G $\alpha$ i2 chimeric G-protein expression plasmid) showed a weak growth response to S-14 (Figure 4A), whereas a much stronger response was observed when p426GPD-rSSTR3 $\Delta$ IC3 was assayed under

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similar conditions (Figure 4B). These results indicate that deletion of a portion of the IC3 improves the function of the SSTR3 in yeast.

#### **Example 5. An IC3 deleted Human Alpha2A Adrenergic Receptor**

As shown in Examples 1-4, deletion of portion of the IC3 of mammalian GPCRs is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. The mutated MARs, CCKBR, and SSTR3 retain all external loops. Transmembrane domains and internal domains other than the IC3 are unchanged. The IC3, found between 5th and 6th membrane spanning helices, was the only domain modified. The bulk of this domain was deleted leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, IC3 of the GPCRs containing the IC3 deletion (IC3 $\Delta$ ) is 44 amino acids in length. The improvement in functional expression may be due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

In order to test the possibility that other IC3 $\Delta$  mutations would also improve functional expression of other GPCRs in yeast, DNA sequences encoding an IC3 $\Delta$  human alpha2A adrenergic receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The IC3 $\Delta$  human alpha2A adrenergic receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 39 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides (GGCCAGGATCCAAAAATGGGCTCCCTGCAGCCGGACGC) (SEQ ID NO: 20) and (CGGGCCCCGCGGGCGCTCGGGGCCCAGACCGTTGGGC) (SEQ ID NO: 21). Fragment 2 contained carboxy terminal coding sequences including 41 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides (CGGGCGACAGCCTGCCGCGGC) (SEQ ID NO: 22) and (AGCGGTCGACTCACACGATCCGCTTCCTGTCCCC) (SEQ ID NO: 23). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length alpha2A adrenergic receptor. Using a conventional lithium acetate

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transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

5 Yeast cells containing the wild type IC3Δ human alpha2A adrenergic receptor were assayed in liquid culture using the alpha adrenergic receptor full agonist UK14304 (RBI) and partial agonist clonidine. The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium. Samples of the cell suspension (180 μl) were dispensed to wells of  
10 sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples of the adrenergic receptor agonist, UK14304 ( $10^{-3}$  -  $10^{-10}$  M). The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell  
15 growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM and were plotted vs. agonist concentration.

The yeast cells containing the wild type IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that this IC3 deletion is functional (Figure 5).

#### **Example 6. Truncation of the Rat Neurotensin NT1 Receptor Causes an Increase in Agonist Sensitivity**

20 In examples 1-4, modification of the third intracellular loop leads to improvement in functional expression of a variety of heterologous GPCRs expressed  
25 in yeast. Agonist induced desensitization of the GPCRs is also mediated in part by GPCR internal domains other than the third intracellular loop, such as the intracellular carboxy-terminal tail.

30 Elimination of the carboxy terminal domains from GPCRs has been shown to improve functional expression in yeast and mammalian cells. Truncation of the carboxy terminal tail of the G protein-coupled alpha mating pheromone receptor expressed in α-mating type yeast cells causes supersensitivity to the presence of

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mating pheromone (*Reneke et al. (1988); Konopka et al (1988)*). Consistent with these observations, a mutated rat neurotensin NT1 receptor (rNTR1) lacking its carboxy terminal tail is resistant to agonist-induced internalization when expressed in mammalian cells (*Hermans et al. (1996)*).

5 To test whether carboxy-terminal truncation improves the functional response of a heterologous GPCR expressed in yeast, the rat NTR1 was modified by deleting the 52 amino acids that constitute the carboxy terminal tail, leaving a shortened receptor 372 amino acids in length. The coding sequences of the wild type and truncated neurotensin NT1 receptor (rNTR1 C-term  $\Delta$ ), were amplified by PCR using  
10 a 5' oligonucleotide primer that specified a common amino-terminal coding sequence (AGTCAGATCTAAGCTT AAAA ATG CAC CTC AAC AGC TCC) (SEQ ID NO: 24) and separate oligos that define the wild type (AGTC AGATCT CTA GTA CAG GGTCTCCC) (SEQ ID NO: 25) and truncated carboxy termini (AGAG AGATCT TTAGCGCCACCCAGGACAAAGGC) (SEQ ID NO: 26). These fragments were  
15 cloned into proximity of the PGK promoter in the yeast expression vector pPGK by standard methods (*Y-S. Kang et al. (1990)*). Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188 that are useful for performing assays of GPCR agonist-stimulated growth, including, specifically, the MPY578fc  
20 cells described in *Pausch et al. (1998)*.

Yeast cells containing the NTR1s were assayed in liquid culture using the NT receptor agonist acetyl neurotensin 8-13 (AcNt8-13). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 2 mM 3-aminotriazole to decrease basal  
25 growth rate. Samples of the cell suspension (180  $\mu$ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20  $\mu$ l of serially-diluted samples ( $10^{-3}$ - $10^{-10}$  M) of AcNt8-13. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD<sub>620</sub> using a microplate reader. Growth rate measurements were obtained during the logarithmic phase of  
30 yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean  $\pm$  SEM and were plotted against agonist

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concentration. As shown in Figure 6, the yeast cells containing the NTR1s produced an agonist-dependent growth response demonstrating that both the wild type and carboxy terminally truncated NTR1s were functional. The growth response of the rNTR1 C-term  $\Delta$  containing cells was dose-dependent giving an EC50 for AcNT8-13 equal to 520 nM. This value is five fold lower than observed for cells expressing the wild type NTR1 (2.1  $\mu$ M). The carboxy terminal deletion has produced a rNTR1 that responds to a lower concentration of NTR agonist improving the sensitivity of the yeast bioassay.

Thus, deletion of a portion of the carboxy terminal intracellular domain of the rat NTR1 has produced a functional GPCR with increased agonist sensitivity when expressed in yeast, suggesting that modification of this internal domain is a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

#### **Example 7. Functional Expression of a Mutated *C. elegans* Serotonin Receptor in Yeast**

Agonists of the G protein-coupled *C. elegans* serotonin receptor (Ce 5HTR) may possess substantial nematocidal activity. These observations suggest that development of a yeast-based HTS for agonists active at Ce 5HTRs may be useful in identifying lead compounds that might be developed into nematocides with a novel mode of action. Preliminary experiments indicated that the wild type Ce 5HTR was non-functional in yeast. Thus, an effort to develop a method for improving the function of the Ce 5HTR in yeast was mounted, via replacement of the Ce 5HTR IC3 with the functional IC3 from the M3 MAR IC3 $\Delta$ .

In order to construct the IC3 replacement, PCR fragments encoding three domains were prepared by standard means. Fragment 1 contains the amino terminal-coding portion of the Ce 5HTR to the intracellular interface of the 5th TMD, amplified by PCR using oligonucleotides (AAAAGATCTAAAATGATCGACGAGACGCTTC) (SEQ ID NO: 27) and (CCGCTTGGTGATCTGACTTCTGGTTTCTGTCCCAGAGGCCTGTAGGCCAGCCAGCTCTTTGGTACGCTTCTCAGTTTCCTTATAGATCTTCCAGTACACGC

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AAATTATTGC) (SEQ ID NO: 28). The second fragment of the Ce 5HTR contains carboxy-terminal coding sequences starting proximal to the intracellular interface of the 6th TMD, amplified by PCR using oligonucleotides (AAACTCGAGTCAATAATCGTGAATAAGGCA) (SEQ ID NO: 29) and (GGCCTACAGGCCTCTGGGACAGAAACCAGAAGTCAGATCACCAAGCGGA AGAGGATGTCGCTCATCAAGGAGAAGAAGGCCGCCAGAACGCTAGCAAT TATTACAGGTAC) (SEQ ID NO: 30). The M3 MAR IC3Δ domain was amplified by PCR as described in Example 1.

These fragments were isolated, mixed and amplified by PCR with 5' (AAAAGATCTAAAATGATCGACGAGACGCTTC) (SEQ ID NO: 27) and 3' (AAACTCGAGTCAATAATCGTGAATAAGGCA) (SEQ ID NO: 29) oligonucleotides. The resulting fragment was digested with appropriate restriction endonucleases and assembled in p426GPD to place the mutated Ce 5HTR under control of the GPD promoter. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. 1998*.

Yeast cells containing the Ce 5HTR were assayed in liquid culture. The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 2 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (200 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 2.0 μl of serially-diluted samples ( $10^{-2}$  -  $10^{-9}$  M) of serotonin (5HT). In similar reactions, the serotonergic antagonists lisuride and mianserin were added to each well at 10 μM. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements were obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM, plotted vs. agonist concentration.  $K_i$  values were

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determined using the equation of Cheng and Pruesoff. (*Y. Cheng et al. (1973)*). As shown in Figure 7, the yeast cells containing the mutated Ce 5HTR containing the M3 MAR IC3 $\Delta$  produced an agonist-dependent growth response, demonstrating that the Ce 5HTR-M3 MAR IC3 $\Delta$  is functional. As expected, the serotonergic antagonists  
5 lisuride and mianserin blocked the growth inducing effect of serotonin, demonstrating that the Ce 5HTR exhibits the expected pharmacological properties when expressed in yeast.

Thus, replacing IC3 of the Ce 5HTR with the functional deleted IC3 from the rat M3 MAR produces a functional chimeric GPCR when expressed in yeast,  
10 indicating that this method of replacing internal domains may be a generalizable method for improving the functional expression of heterologous GPCRs in cell-based assays, such as yeast assays.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.  
15 It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

The references cited herein are specifically incorporated by reference in their entirety.



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We claim:

1. An isolated nucleic acid sequence having a sequence encoding a modified G protein-coupled receptor (GPCR), wherein the modification comprises a mutation in an intracellular domain of the G protein-coupled receptor and results in an improved functional response in a cell-based assay, and wherein the modified G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, a somatostatin receptor, an alpha 2A adrenergic receptor, and a serotonin receptor.
2. The nucleic acid sequence according to claim 1, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist.
3. The nucleic acid according to claim 2, wherein the modification results in improved coupling between the receptor and a heterotrimeric G protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.
4. The nucleic acid according to claim 1, wherein the mutation is a deletion.
5. The nucleic acid according to claim 4, wherein the deletion is a point mutation.
6. The nucleic acid according to claim 4, wherein the deletion is in the third intracellular loop of the G protein-coupled receptor.
7. The nucleic acid according to claim 6, wherein the G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, and an alpha 2A adrenergic receptor.
8. The nucleic acid sequence according to claim 1, wherein the serotonin receptor is Ce 5HTR.
9. The nucleic acid sequence according to claim 1, wherein the muscarinic acetylcholine receptor is a rat M3 muscarinic acetylcholine receptor or a *D. melanogaster* muscarinic acetylcholine receptor.
10. The nucleic acid sequence according to claim 1, wherein the cholecystokinin CCKB receptor is a rat cholecystokinin CCKB receptor.

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11. The nucleic acid sequence according to claim 1, wherein the somatostatin receptor is a rat somatostatin receptor subtype 3.

12. The nucleic acid sequence according to claim 1, wherein the alpha 2A adrenergic receptor is a human alpha 2A adrenergic receptor.

13. A modified G protein-coupled receptor encoded by the nucleic acid sequence according to claim 1.

14. The G protein-coupled receptor according to claim 13, wherein the deleted third intracellular loop is 44 amino acids in length.

15. A mutant G protein-coupled receptor encoded by the nucleic acid sequence according to claim 10, wherein a sequence Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO:15) is deleted from the third intracellular loop of the mutant G protein-coupled receptor.

16. An isolated nucleic acid having a sequence encoding a chimeric G protein-coupled receptor, wherein the chimeric G protein-coupled receptor comprises a modified intracellular domain of a G protein-coupled receptor that confers an improved functional response to the chimeric G protein-coupled receptor in a cell-based assay.

17. The nucleic acid according to claim 16, wherein the modified intracellular domain is the third intracellular loop.

18. A vector comprising the nucleic acid sequence according to claim 1 or 16.

19. A host cell transformed with a vector according to claim 10.

20. The host cell according to claim 19, further comprising a plasmid comprising an inducible reporter gene.

21. The host cell according to claim 19, wherein the host cell is a eukaryotic cell.

22. The host cell according to claim 21, wherein the eukaryotic cell is a yeast cell.

23. The host cell according to claim 22, further comprising a plasmid comprising an inducible reporter gene.

24. The host cell according to claim 23, wherein the reporter gene is a green fluorescent protein.

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25. The host cell according to claim 24, wherein the green fluorescent protein is operably linked to a *FUS2* promoter.

26. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the host cell according to claim 19 to a test compound; and
- (b) measuring the effect of the test compound on cell growth.

27. A host cell comprising a heterologous G protein-coupled receptor, wherein the G protein-coupled receptor has a modification that results in an improved functional response of the G protein-coupled receptor in a cell-based assay.

28. The host cell according to claim 27, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist

29. The host cell according to claim 28, wherein the modification results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the heterologous G protein-coupled receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.

30. The host cell according to claim 27, wherein the host cell is a eukaryotic cell.

31. The host cell according to claim 30, wherein the heterologous G protein-coupled receptor is modified at an intracellular domain.

32. The host cell according to claim 31, wherein the intracellular domain is the third intracellular domain.

33. The host cell according to claim 27, wherein the heterologous G protein-coupled receptor is modified at the carboxy terminal tail of the G protein-coupled receptor.

34. The host cell according to claim 31 or 33, wherein the host cell is yeast.

35. The host cell according to claim 34, wherein the modified G protein-coupled receptor is a neurotensin receptor.

36. The host cell according to claim 35, wherein the neurotensin receptor is a rat neurotensin NT1 receptor.

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37. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the host cell according to claim 27 to a test compound; and
- (b) measuring the effect of the test compound on yeast cell growth.

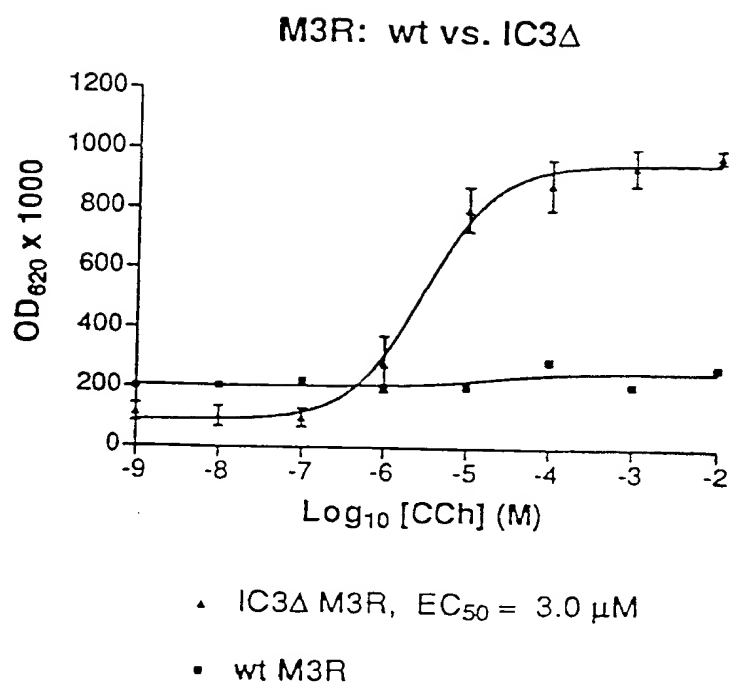
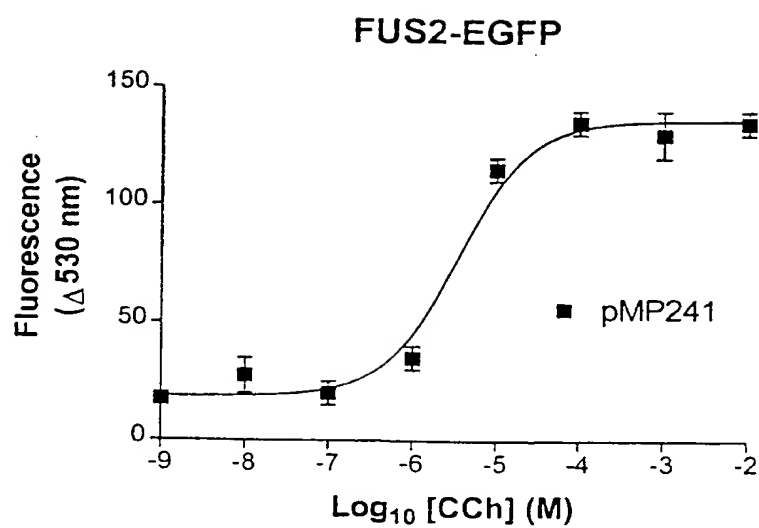


FIG. 1A

**FIG. 1B**



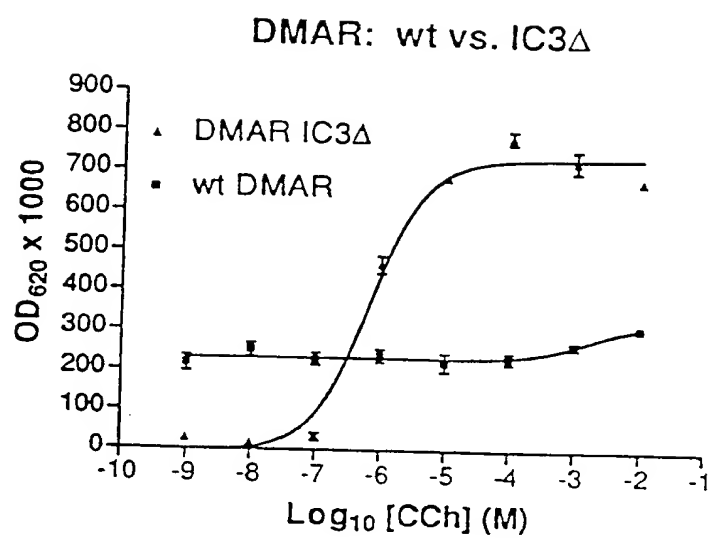
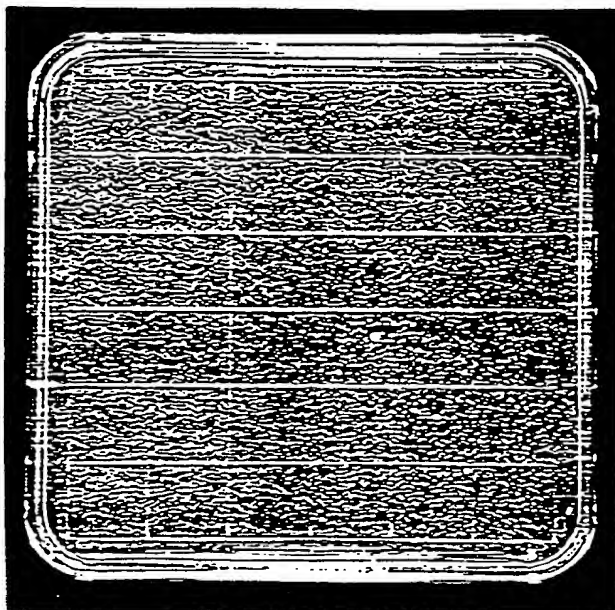
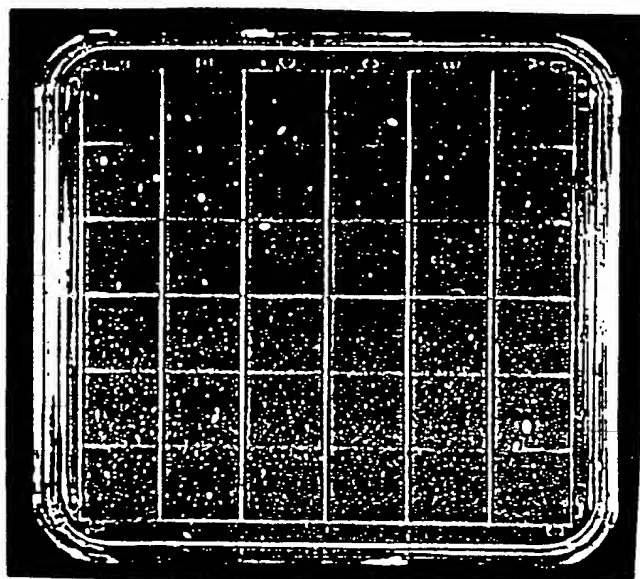


FIG. 2

**A**

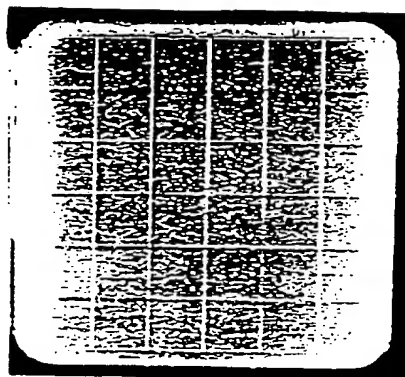


**B**

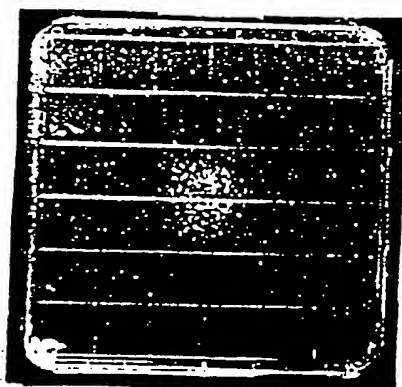


**FIG. 3**

**A**



**B**



**FIG. 4**

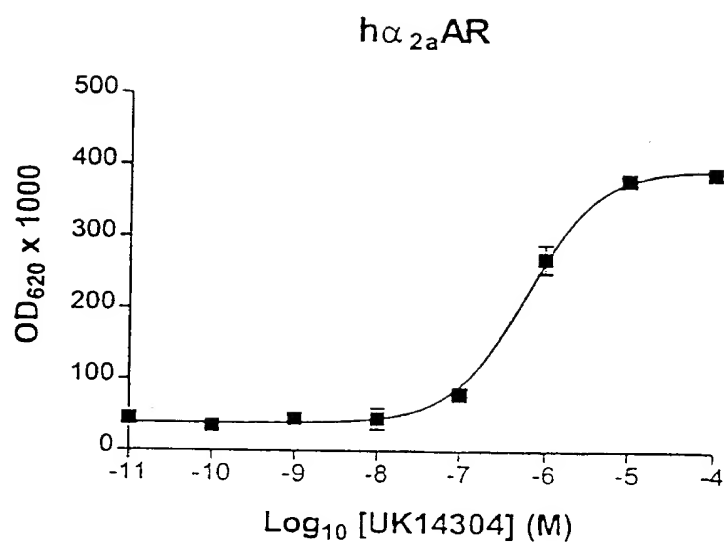


FIG. 5

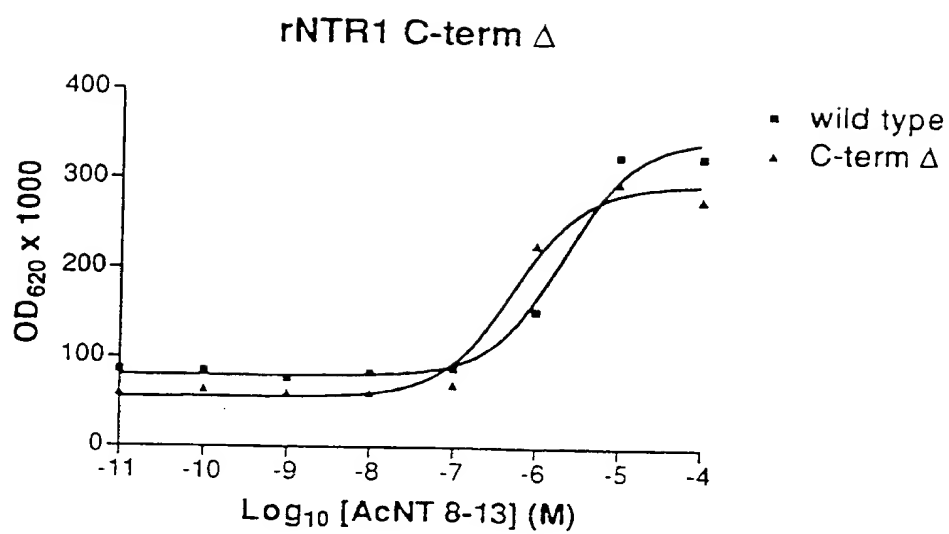


FIG. 6

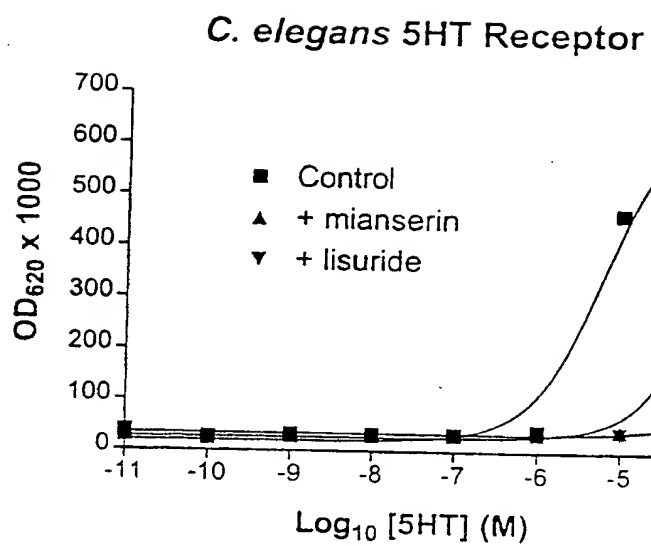


FIG. 7

# METHODS FOR IMPROVING THE FUNCTION OF HETEROLOGOUS G PROTEIN-COUPLED RECEPTORS

## STATEMENT OF RELATED APPLICATIONS

This application hereby claims the benefit of United States provisional application Serial No. 60/098,704 filed September 1, 1998. The entire disclosure of this provisional application is relied upon and incorporated by reference herein.

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## FIELD OF THE INVENTION

This invention relates to mutant G protein-coupled receptors with improved G-protein coupling and receptor response, host cells expressing such receptors, vectors useful for making such cells, and methods of making and using same.

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## BACKGROUND OF THE INVENTION

The actions of many extracellular signals, such as neurotransmitters, hormones, odorants, and light, are mediated by a triad of proteins which has been identified in organisms from yeast to mammals. This triad consists of a receptor, coupled to a trimeric guanine nucleotide-binding regulatory protein (G protein), which in turn is coupled to a cellular effector. These receptors have seven transmembrane domains and are named for their association with the G protein as "G protein-coupled receptors" ("GPCRs").

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The regulatory G proteins are comprised of three subunits: a guanylnucleotide binding  $\alpha$  subunit; a  $\beta$  subunit; and a  $\gamma$  subunit. *B.R. Conklin and H.R. Bourne (1993)*. G proteins cycle between two forms, depending on whether GDP or GTP is bound to the  $\alpha$  subunit. When GDP is bound, the G protein exists as a heterotrimer, the  $G\alpha\beta\gamma$  complex. When GTP is bound, the  $\alpha$  subunit dissociates, leaving a  $G\beta\gamma$  complex. Importantly, when a  $G\alpha\beta\gamma$  complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of disassociation of the bound  $G\alpha$  subunit from the  $G\beta\gamma$  complex increases. The free  $G\alpha$  subunit and  $G\beta\gamma$  complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways. Examples of these downstream cellular effector proteins include, among others, adenylate cyclases, ion channels, and phospholipases. This

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fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. *H.G. Dohlman et al. (1991)*.

Because of their ubiquitous nature in important biochemical pathways, the G protein-coupled receptors represent important targets for new therapeutic drugs. In turn, the discovery of such drugs will necessarily require screening assays of high specificity and throughput termed high-throughput screening (HTS) assays. Screening assays utilizing microorganisms, such as yeast strains, genetically modified to accommodate functional expression of the G protein-coupled receptors offer significant advantages in research involving ligand binding to numerous receptors implicated in various disease states.

However, microorganisms transformed with wild-type receptors may perform poorly in growth assays, exhibiting, for example, the inability to interact with the heterotrimeric G protein, inappropriate localization and/or desensitization. Many GPCRs are phosphorylated in response to chronic and persistent agonist stimulation which often leads to desensitization followed by sequestration or internalization of the receptors. Desensitization of GPCRs causes uncoupling from interaction with heterotrimeric G proteins. This process is mediated by a variety of regulatory receptor protein kinases, including G protein-coupled receptor kinases (GRK), protein kinase A (PKA), protein kinase C (PKC), and casein kinases (CK). Internalization involves removal of GPCRs from the plasma membrane via receptor-mediated endocytosis. Internalized receptors may be recycled back to the cell surface, or delivered to a lysosomal/vacuolar compartment for degradation. The ubiquitin-mediated degradative pathway is also involved in this process. The ultimate result of receptor phosphorylation and sequestration/internalization is often cell growth arrest, which significantly reduces the utility of the genetically modified microorganism in screening assays.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide modified G protein-coupled receptors that function well in high throughput screening assays, implemented in any eukaryotic cell, preferably yeast cells. Thus, a first aspect of the present invention is



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directed to nucleotide sequences encoding a G protein-coupled receptor which has been modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery, and/or to appropriately localize to the plasma membrane. In preferred embodiments, such modifications lead to improved agonist-stimulated growth-promoting ability. One specific modification of the nucleotide sequence encoding a G protein-coupled receptor encompassed by this invention is a mutation in any intracellular domain or membrane region proximal to internal domains. The mutation may be a deletion, including, for example, a point mutation.

This invention is also directed to chimeric GPCRs in which intracellular domains of heterologous GPCRs that provide favorable G protein coupling properties or domains not subject to the yeast cell desensitization and/or sequestration/internalization machinery are used to replace comparable domains in GPCRs of interest. This invention also relates to the modified nucleotide sequences encoding the chimeric GPCRs, to expression vectors comprising the modified nucleotide sequences, and to host cells transformed therewith.

An additional aspect of this invention is an improved method of assaying compounds to determine effects of ligand binding to the mutant or chimeric GPCRs of this invention by measuring the effect of the test compound on cell growth. The mutant GPCRs prevent or reduce the rate of cell growth arrest due to chronic and persistent agonist stimulation, thereby decreasing the number of false negatives that occur with prior art screening methods and/or increasing the sensitivity of the bioassay.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**FIGURE 1A** depicts the results of liquid culture assays on yeast cells containing the rat M3 muscarinic acetylcholine receptor (MAR) using MAR agonist carbachol (CCh). Yeast cells containing a M3 MAR with a deletion in the third intracellular loop (IC3Δ) produced an agonist-dependent growth response, while the wild type MAR did not, indicating that the M3 MAR IC3Δ is functional GPCR.

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**FIGURE 1B** depicts the results of liquid culture fluorescence induction assays on yeast cells containing the rat M3 MAR IC3Δ and the FUS2-GFP reporter plasmid using the MAR agonist carbachol (CCh). A dose-dependent increase in the expression of the green fluorescent protein is observed in response to CCh activation of the M3 MAR IC3Δ expressed in yeast.

**FIGURE 2** depicts the results of liquid culture assays on yeast cells containing the *Drosophila* muscarinic acetylcholine receptor using the MAR agonist carbachol (CCh). Yeast cells containing a mutated *Drosophila* MAR containing the M3 MAR IC3Δ produced an agonist-dependent growth response while the wild type *Drosophila* MAR lacked an agonist-dependent yeast cell growth response.

**FIGURE 3** depicts the results of an agar-based plate bioassay. **FIGURE 3A** shows a robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor. **FIGURE 3B** shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of a portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

**FIGURE 4** depicts yeast cells transformed with rSSTR3 and with rSSTR3ΔIC3. **FIGURE 4A** demonstrates that yeast cells containing p426GPD-rSSTR3 show a weak response to somatostatin (S-14). **FIGURE 4B** demonstrates a much stronger response by yeast cells containing p426GPD-rSSTR3ΔIC3 assayed under similar conditions.

**FIGURE 5** depicts the results of liquid culture assays on yeast cells containing wild type IC3Δ human alpha2A adrenergic receptor using the alpha adrenergic receptor full agonist UK14304. Yeast cells containing the wild type and IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that the IC3 deletion is functional.

**FIGURE 6** depicts the results of liquid culture assays on yeast cells containing wildtype and carboxy terminally truncated rat NT1-neurotensin receptors using the neurotensin receptor agonist AcNT8-13. Truncation of the rat NT1-neurotensin receptor produces an agonist-dependent growth response that is more sensitive than that observed with the wildtype receptor.

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**FIGURE 7** depicts the results of liquid culture assays on yeast cells containing the *C. elegans* serotonin receptor using serotonin (5HT) to stimulate yeast cells growth. Yeast cells containing a mutated *C. elegans* serotonin receptor containing the M3 MAR IC3Δ produced a 5HT-dependent growth response. The growth response was blocked by addition of the serotonin receptor antagonists lisuride and mianserin.

### DETAILED DESCRIPTION OF THE INVENTION

#### Modified G Protein-Coupled Receptors

Nucleotide sequences encoding G protein-coupled receptors may be modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery. Such modifications lead to improved agonist-stimulated yeast cell growth-promoting ability. The improvement of GPCR-G protein coupling and/or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell provides a means to improve the function of wildtype heterologous GPCRs that fail to stimulate a useful yeast cell growth response. Thus, GPCRs that fail to function in their wild type form may be made to work by the methods of this invention.

The improvement of GPCR-G protein coupling and or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell may be assessed by using routine techniques, such as those described in the Examples set forth below or known to those of skill in the art. For example, improvement of the function of a mutated GPCR over wild type may be quantified as an increase in the signal-to-noise ratio and/or in the sensitivity of the liquid bioassay. The signal-to-noise ratio is determined by comparing the agonist-induced growth rate to the growth rate observed in the absence of agonist. A statistically-significant increase in the signal-to-noise ratio resulting from agonist-stimulation of a mutated GPCR over similar values obtained from cells containing a wild type receptor indicates that the function of the mutated GPCR has been improved.

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The sensitivity of the liquid bioassay is defined as the agonist concentration necessary to produce a half-maximal growth rate (ED50 or EC50). The sensitivity of the bioassay is increased if a mutated GPCR produces a half-maximal growth rate at an agonist concentration that is less than that required by the wild type GPCR.

5 Similarly, the more qualitative agar based bioassay will reflect increases in signal-to-noise ratio and/or sensitivity due to agonist stimulation of mutated GPCRs. In the agar based bioassay, signal-to-noise ratio increases are determined by comparing the extent of growth within the agonist induced growth zone resulting from stimulation of mutated and wild type receptor. The sensitivity of the bioassay is  
10 proportional to the radius of the growth zone. Since applied compounds diffuse radially from the site of application to the agar, agonist concentration varies with the square of the radius of the growth zone. Thus, a larger zone of growth in response to agonist activation of mutated GPCRs reflects an increase in sensitivity.

Any G protein-coupled receptor may be employed in practicing this invention.  
15 Examples of such receptors include, but are not limited to, adenosine receptors, somatostatin receptors, dopamine receptors, cholecystokinin receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, serotonin receptors, vasopressin receptors, melanocortin receptors, and neurotensin receptors.  
20 In certain preferred embodiments, the receptor is a muscarinic acetylcholine receptor and more preferably, the muscarinic acetylcholine receptor is of the M3 subtype.

Similarly, any suitable host cell may be transformed with the nucleotide sequences encoding the modified G protein-coupled receptors of this invention. Examples of suitable host cells are yeast cells, mammalian cells, insect cells, and  
25 bacterial cells. Preferably, the host cells are yeast cells.

One generalizable method for improving the function of a GPCR expressed in a host cell is by modification or elimination of intracellular domains of the GPCR, such as the third intracellular loop (IC3) sequences of the G protein-coupled receptor. Because the desensitization and internalization machinery acts upon the intracellular  
30 domains of the GPCR, elimination of the intracellular domains of the GPCRs produces a more stable receptor expression. This has been demonstrated in

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experiments conducted in mammalian cells. Muscarinic acetylcholine receptors, including the M3 subtype, lacking a domain of their third intracellular loop thought to be involved in receptor internalization, are maintained in the plasma membrane to a greater extent than their wild type counterparts. *Moro et al. (1993)*.

Representative embodiments of the invention are described in more detail in the following examples.

**Example 1. Functional Expression of A Mutated Rat M3 Muscarinic Acetylcholine Receptor (MAR) in Yeast**

The third intracellular loops of GPCRs are thought to interact with and participate in the activation of G proteins upon agonist binding. *J. Wess (1997)*. Mutations in IC3 of the yeast mating pheromone receptors, Ste2 and Ste3 have profound effects on coupling the G proteins. *C. Boone et al. (1993)* and *C. Clark et al. (1994)*. Importantly, deletion of portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in mammalian cells with retention of full ability to couple to the heterotrimeric G protein, Gq ( $G\alpha\beta\gamma$ ). The mutated M3 MAR retains all external loops. Transmembrane domains (TMDs) and internal domains other than the IC3 are unchanged. The IC3, found between 5th and 6th membrane spanning helices, was the only domain modified. The bulk of this domain, 96 amino acids in the center of the IC3 (Ala273-Lys469), were deleted, leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, the third intracellular loop of the MAR containing the IC3 deletion (IC3 $\Delta$ ) is 44 amino acids in length, compared to 240 amino acids in the IC3 of wild type M3 MAR. The improvement in functional expression may due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

In order to test the possibility that this IC3 $\Delta$  mutation would also improve functional expression in yeast, the DNA sequences encoding the wild type and IC3 $\Delta$  rat M3 MARs were cloned into proximity to the glycerol-phosphate dehydrogenase promoter in the yeast expression plasmid, p426GPD, by standard methods. Rat M3

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MAR sequences were amplified by PCR using oligonucleotides containing 5' *Bgl*II (AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *Xho*I (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. The resulting PCR product was digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. To form the rat M3 IC3Δ, three M3 MAR fragments were amplified by PCR. An amino-terminal coding fragment was amplified using oligonucleotides containing 5' *Bgl*II (AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *Age*I (ATAGTCATGATGGTG ACCGGT ATGTAAAAGGCAGCGATC) (SEQ ID NO: 3) sites. A carboxy-terminal coding fragment was amplified using oligonucleotides containing 5' *Pml*II (GCCTTCATCAT CACGTG GACCCCCTACACC) (SEQ ID NO: 4) and 3' *Xho*I (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. An IC3 coding fragment was amplified using oligonucleotides containing 5' *Age*I (CGATCGCTGCCTTTTACTT ACCGGT CACCATCATGACTAT) (SEQ ID NO: 5) and 3' *Pml*II (GTTGTAGGGGGTGC CACGTG ATGATGAAGGC) (SEQ ID NO: 6) sites using the M3 IC3Δ sequence. *J. Wess (1997)*. The resulting PCR products were digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. Plasmids were confirmed by restriction endonuclease mapping and DNA sequencing. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, incorporated herein by reference, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

Yeast cells containing the MARs were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples ( $10^{-1}$ - $10^{-8}$  M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600

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rpm). Growth was monitored by recording increases in  $OD_{620}$  using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean  $\pm$  SEM and were plotted vs. agonist concentration. As shown in Figure 1, the yeast cells containing the M3 MAR IC3 $\Delta$  produced an agonist-dependent growth response, demonstrating that the M3 MAR IC3 $\Delta$  is functional, while the wild type MAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth. The growth response of the M3 MAR IC3 $\Delta$  containing cells was dose-dependent giving an  $EC_{50}$  for carbachol (CCh) equal to 3  $\mu$ M. This value matches the  $K_D$  for CCh obtained in HEK cells (7.9  $\mu$ M) and the  $EC_{50}$  for CCh induced IP<sub>3</sub> (inositol triphosphate) accumulation (4.0  $\mu$ M), suggesting that the M3 MAR IC3 $\Delta$  retains the expected pharmacological properties when expressed in yeast cell membranes. Further, the growth response is blocked by the MAR-specific antagonist, atropine (At).

Alternatively, the response to CCh by yeast cells containing the M3 MAR IC3 $\Delta$  may be observed by measuring the agonist-induced increase in fluorescent emission from a green fluorescent protein reporter gene whose expression is stimulated by MAR agonists. Green fluorescent protein (GFP) is a protein from *Aequorea* that is intrinsically fluorescent when expressed in yeast cells. The fluorescence from GFP is detectable in live yeast cells, making it possible to measure the level of its expression without any deleterious treatment of the yeast cells. This feature is particularly advantageous in the reporter gene assays that do not require additional steps to permit its detection. An inducible reporter gene that is useful in detecting the agonist-activation of heterologous GPCRs expressed in yeast utilizes transcriptional promoters that are activated by the mating signal transduction pathway. One such promoter is the *FUS2* promoter. In the absence of agonist stimulation, little or no expression of the Fus2 protein or any other protein whose expression is directed by the *FUS2* promoter is detectable. After treatment with agonist, transcription from the *FUS2* promoter is induced up to 700 fold, leading to substantial increases in Fus2 expression or in the expression of any gene product whose expression is placed under control of the *FUS2* promoter. Thus, yeast cell fluorescence resulting from GFP

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expression under the control of the *FUS2* promoter from a *FUS2*-GFP reporter gene is only observed after agonist activation of a heterologous GPCR.

In order to produce a GFP reporter gene, DNA sequences encoding the enhanced GFP (EGFP, Clontech), *FUS2* promoter and *FUS2* transcriptional terminator sequences were amplified by PCR. The fragments were assembled into the centromere containing plasmid pRS414 so as to place EGFP expression under control of the pheromone responsive *FUS2* promoter in the centromere containing plasmid pRS414, producing plasmid pMP241. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188, that are useful for performing assays of GPCR agonist-stimulated growth of cells containing the M3 MAR IC3Δ. Specifically, the plasmids were introduced into the MPY578fc cells described in Pausch *et al.* (1998).

Yeast cells containing the M3 MAR IC3Δ and the *FUS2*-EGFP reporter plasmid were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were washed and diluted 5 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples ( $10^{-1}$ - $10^{-8}$  M) of CCh. The plates were incubated at 30°C for 6 hours with agitation (600 rpm). Stimulation of the *FUS2*-EGFP reporter gene expression was monitored by recording increases in emission at 530 nm after excitation with 480 nm light using a fluorescence microplate reader. Assays were conducted in duplicate and measurements obtained during the logarithmic phase of yeast cell growth. Fluorescence emission measurements were analyzed using GraphPad Prism and were presented as the mean ± SEM and were plotted vs. agonist concentration. As shown in Figure 1B, the yeast cells containing the M3 MAR IC3Δ produced a dose dependent increase in fluorescence emission, consistent with increased expression of the EGFP from the agonist inducible *FUS2*-GFP reporter gene construct. The EC<sub>50</sub> for CCh stimulation of fluorescence emission is 4 μM, identical to values obtained in the growth assay.



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Thus, deletion of a portion of the IC3 of the rat M3 MAR has produced a functional GPCR when expressed in yeast, suggesting that modification of internal domains may be a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

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**Example 2. Functional Expression of a Mutated *D. melanogaster* Muscarinic Acetylcholine Receptor in Yeast**

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Agonist of the G protein-coupled insect muscarinic acetylcholine receptors (MARs) possess substantial insecticidal and miticidal activity. *M.R. Dick et al. (1997)*. These observations suggest that development of a yeast-based high throughput screen (HTS) for agonists active at insect MARs may be useful in identifying lead compounds that might be developed into insecticides with novel mode of action. Preliminary experiments indicate that the wild type *D. melanogaster* MAR (DMAR), an insect G protein-coupled receptors (GPCRs), is non-functional in yeast. Thus, an effort to develop a method for improving the function of the DMAR in yeast was mounted, via replacement of the DMAR IC3 with the functional IC3 from the M3 MAR IC3Δ.

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In insect cells, the DMAR interacts with the heterotrimeric Gq protein leading to an increase in intracellular calcium in response to muscarinic agonists. One potential explanation for the inactivity of the DMAR in yeast is an inability to efficiently couple to the yeast heterotrimeric G protein. Thus, to devise a method to improve the DMAR function in yeast, selected mutations in the GPCRs that serve to improve functional expression and coupling to the heterotrimeric G protein were examined.

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In order to construct the IC3 replacement, PCR fragments encoding three domains were prepared by standard means. Fragment 1 consisted of the amino terminal coding portion of the *Drosophila* MAR up to an *AgeI* site within the 5th TMD, amplified by PCR using oligonucleotides (AAA AGATCT AAA ATG TACGGAAACCAGACGAAC) (SEQ ID NO: 7) and (CCA GTA GAG GAA GCACATGATGGTC AGGCCT AAG TAG AAG GCG GCC AGT GC) (SEQ ID NO: 8). The second fragment of the DMAR was composed of carboxy terminal

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coding sequences starting with a *PmlI* site in the 6th TMD, amplified by PCR using oligonucleotides (TTCATCATCACGTGGACTCCGTACAACATC) (SEQ ID NO: 9) and (AAA CTCGAG TTATCTAATTGTAGACGCGGC) (SEQ ID NO: 10). The M3 MAR IC3Δ domain was amplified as an *AgeI*-*PmlI* fragment with coding sequence in frame with fragments 1 and 2, described in Example 1. These fragments were assembled in plasmid p426GPD to place the mutated DMAR under control of the GPD promoter. The wild type DMAR was cloned into the expression vector, pMP3, described in United States Patent 5,691,188. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

Yeast cells containing the DMAR and the plasmid containing the wild type DMAR were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8, medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples ( $10^{-1}$  -  $10^{-8}$  M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM and were plotted vs. agonist concentration. As shown in Figure 2, the yeast cells containing the mutated DMAR, i.e., the M3 MAR IC3Δ, produced an agonist-dependent growth response, demonstrating that the DMAR-M3 MAR IC3Δ is functional. The wild type DMAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth.

Thus, replacing IC3 of the DMAR with the functional deleted IC3 from the rat M3 MAR produces a functional chimeric GPCR when expressed in yeast, indicating that this method of replacing internal domains may be a generalizable method for

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improving the functional expression of heterologous GPCRs in cell-based assays, such as yeast assays.

**Example 3. Functional Expression of a Mutated Rat Cholecystokinin CCKB Receptor in Yeast**

As shown in Examples 1 and 2, deletion of portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. In order to test the possibility that this IC3 $\Delta$  mutation would also improve functional expression of other GPCRs in yeast, the DNA sequences encoding the rat wild type and IC3 $\Delta$  cholecystokinin CCKB receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The wild type CCKBR was amplified by PCR using oligonucleotides (ACTTAGATCAAAAAATGGAGCGCTCAAGCTGAACCG) (SEQ ID NO: 11) and (TCCCGTCGACTCAGCCAGGCCCCAGTGTGCTG) (SEQ ID NO: 12). The IC3 $\Delta$  cholecystokinin CCKB receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 22 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides (ACTTAGATCAAAAAATGGAGCGCTCAAGCTGAACCG) (SEQ ID NO: 11) and (CGAGGGCCAGGGACTGGCCCCGGCCGGGCCCCGGCTTTGGGTCTCG) (SEQ ID NO: 13). Fragment 2 contained carboxy terminal coding sequences including 22 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides (TCCCGTCGACTCAGCCAGGCCCCAGTGTGCTG) (SEQ ID NO: 12) and (CGAGACCCAAAGCCGGGCCCCGGCCGGGCCCCAGTCCCTGGCCCTCG) (SEQ ID NO: 14). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length CCKB receptor. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

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Yeast strains containing wild type and IC3Δ cholecystokinin CCKB receptor were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil (SCD-ura) medium. In this agar-based plate bioassay, molten (50°C) SCD-ura-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH<sub>4</sub>OH prior to autoclaving) containing 0.5 mM AT (3-aminotriazole) was inoculated with the overnight culture (2 x 10<sup>4</sup> cells/ml) and poured into square (9 x 9 cm) petri plates. Solutions of CCK agonists in DMSO (1 mM, 10 μl) were applied to the surface of the solidified agar (Upper left: CCK8S; upper right, CCK8US; lower left, CCK5; lower right, CCK4). Compounds applied to the surface of the plate diffused radially from the site of application and bound to CCKB receptors expressed on the surface of cells embedded in the agar, resulting in induction of *FUS1-HIS3* expression. The responding cells formed a dense growth zone readily detectable over the limited growth of cells observed in response to basal *FUS1-HIS3* expression. Plates were incubated at 30°C for 3 days (Figure 3). Figure 3A demonstrates the robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor, while Figure 3B shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

#### **Example 4. Functional Expression of a Mutated Rat Somatostatin Receptor (SSTR) in Yeast**

The third intracellular loop participates in many GPCR functions, including G protein coupling, desensitization and interaction with diverse modifying proteins. Somatostatin receptors are encoded in five subtypes, labeled SSTR1-5. Several amino acids are found in the third intracellular loop of the SSTR3 subtype, but not in the equivalent region of SSTR2 subtype. Since SSTR2 functions efficiently in yeast, deletion of those amino acids from IC3 may impart this functional efficiency upon SSTR3. Thus, 8 amino acids, Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO: 15), were deleted from the third intracellular loop of the rSSTR3 cDNA, enabling more efficient receptor signaling in yeast.

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Rat SSTR3 sequences were amplified by PCR using oligonucleotides containing 5' BglII and 3' XhoI sites. The resulting PCR product of approximately 1.3 kb was digested with BglII and XhoI, purified and inserted between the BamHI and XhoI sites in p426GPD to generate the plasmid p426GPD-rSSTR3. Recombinant plasmids were confirmed by restriction endonuclease digestion and DNA sequencing.

Standard PCR reactions were used to amplify the rSSTR3 cDNA to yield two PCR fragments that have 36 bp overlap as follows. PCR insert A of approximate size 750 bp was generated using the 5' Bgl oligonucleotide (AAAAAGATCT AAAATGGCCA CTGTTACCTA T) (SEQ ID NO: 16) and the 3' oligonucleotide CTCAGAGCGG CGTCGCCGCT GACACGAGGG CGCCCG (SEQ ID NO: 17). PCR insert B of approximate size 530 bp was generated using the 5' oligonucleotide GCGCCCTCGT GTCAGCGGCG ACGCCGCTCT GAG (SEQ ID NO: 18) and the 3' XhoI oligonucleotide (AAAACTCGAG TTACAGATGG CTCAGTGTGC T) (SEQ ID NO: 19). PCR fragments A and B were gel purified, annealed and amplified by PCR using the flanking 5' BglII and 3' XhoI oligonucleotides to yield the ~1.3 kb rSSTR3 $\Delta$ IC3 PCR product. Following purification and digestion with BglII-XhoI, the rSSTR3 $\Delta$ IC3 insert was ligated into BamHI-XhoI sites of p426GPD to generate the plasmid p426GPD-rSSTR3 $\Delta$ IC3. Restriction mapping and DNA sequencing confirmed correct reading frame and sequence.

Yeast cells of the type useful for expression of GPCRs, described in United States Patent 5,691,188, were transformed with p426GPD-rSSTR3 and p426GPD-rSSTR3 $\Delta$ IC3, using standard procedures. The cells (i.e., the LY296 cells, described in *Price et al. (1995)*) were assayed using the agar-based bioassay format described in Example 3. Samples (10  $\mu$ l) of Somatostatin (S-14, 1mM) were applied to the surface of the selective agar medium containing the yeast cells expressing the SSTR3. The plates were incubated for 3 days at 30°C. Yeast cells transformed with p426GPD-rSSTR3 along with pLP82 (containing a G $\alpha$ 1/G $\alpha$ i2 chimeric G-protein expression plasmid) showed a weak growth response to S-14 (Figure 4A), whereas a much stronger response was observed when p426GPD-rSSTR3 $\Delta$ IC3 was assayed under

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similar conditions (Figure 4B). These results indicate that deletion of a portion of the IC3 improves the function of the SSTR3 in yeast.

#### **Example 5. An IC3 deleted Human Alpha2A Adrenergic Receptor**

As shown in Examples 1-4, deletion of portion of the IC3 of mammalian GPCRs is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. The mutated MARs, CCKBR, and SSTR3 retain all external loops. Transmembrane domains and internal domains other than the IC3 are unchanged. The IC3, found between 5th and 6th membrane spanning helices, was the only domain modified. The bulk of this domain was deleted leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, IC3 of the GPCRs containing the IC3 deletion (IC3 $\Delta$ ) is 44 amino acids in length. The improvement in functional expression may be due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

In order to test the possibility that other IC3 $\Delta$  mutations would also improve functional expression of other GPCRs in yeast, DNA sequences encoding an IC3 $\Delta$  human alpha2A adrenergic receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The IC3 $\Delta$  human alpha2A adrenergic receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 39 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides (GGCCAGGATCCAAAAATGGGCTCCCTGCAGCCGGACGC) (SEQ ID NO: 20) and (CGGGCCCCGCGGGCGCTCGGGGCCAGACCGTTGGGC) (SEQ ID NO: 21). Fragment 2 contained carboxy terminal coding sequences including 41 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides (CGGGCGACAGCCTGCCGCGGC) (SEQ ID NO: 22) and (AGCGGTCGACTCACACGATCCGCTTCCTGTCCCC) (SEQ ID NO: 23). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length alpha2A adrenergic receptor. Using a conventional lithium acetate

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transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)*.

5 Yeast cells containing the wild type IC3Δ human alpha2A adrenergic receptor were assayed in liquid culture using the alpha adrenergic receptor full agonist UK14304 (RBI) and partial agonist clonidine. The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium. Samples of the cell suspension (180 μl) were dispensed to wells of  
10 sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples of the adrenergic receptor agonist, UK14304 ( $10^{-3}$  -  $10^{-10}$  M). The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell  
15 growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM and were plotted vs. agonist concentration.

The yeast cells containing the wild type IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that this IC3 deletion is functional (Figure 5).

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#### **Example 6. Truncation of the Rat Neurotensin NT1 Receptor Causes an Increase in Agonist Sensitivity**

In examples 1-4, modification of the third intracellular loop leads to  
25 improvement in functional expression of a variety of heterologous GPCRs expressed in yeast. Agonist induced desensitization of the GPCRs is also mediated in part by GPCR internal domains other than the third intracellular loop, such as the intracellular carboxy-terminal tail.

Elimination of the carboxy terminal domains from GPCRs has been shown to  
30 improve functional expression in yeast and mammalian cells. Truncation of the carboxy terminal tail of the G protein-coupled alpha mating pheromone receptor expressed in α-mating type yeast cells causes supersensitivity to the presence of

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mating pheromone (*Reneke et al. (1988); Konopka et al (1988)*). Consistent with these observations, a mutated rat neurotensin NT1 receptor (rNTR1) lacking its carboxy terminal tail is resistant to agonist-induced internalization when expressed in mammalian cells (*Hermans et al. (1996)*).

5 To test whether carboxy-terminal truncation improves the functional response of a heterologous GPCR expressed in yeast, the rat NTR1 was modified by deleting the 52 amino acids that constitute the carboxy terminal tail, leaving a shortened receptor 372 amino acids in length. The coding sequences of the wild type and truncated neurotensin NT1 receptor (rNTR1 C-term  $\Delta$ ), were amplified by PCR using  
10 a 5' oligonucleotide primer that specified a common amino-terminal coding sequence (AGTCAGATCTAAGCTT AAAA ATG CAC CTC AAC AGC TCC) (SEQ ID NO: 24) and separate oligos that define the wild type (AGTC AGATCT CTA GTA CAG GGTCTCCC) (SEQ ID NO: 25) and truncated carboxy termini (AGAG AGATCT TTAGCGCCACCCAGGACAAAGGC) (SEQ ID NO: 26). These fragments were  
15 cloned into proximity of the PGK promoter in the yeast expression vector pPGK by standard methods (*Y-S. Kang et al. (1990)*). Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188 that are useful for performing assays of GPCR agonist-stimulated growth, including, specifically, the MPY578fc  
20 cells described in *Pausch et al. (1998)*.

Yeast cells containing the NTR1s were assayed in liquid culture using the NT receptor agonist acetyl neurotensin 8-13 (AcNt8-13). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 2 mM 3-aminotriazole to decrease basal  
25 growth rate. Samples of the cell suspension (180  $\mu$ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20  $\mu$ l of serially-diluted samples ( $10^{-3}$ - $10^{-10}$  M) of AcNt8-13. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD<sub>620</sub> using a microplate reader. Growth rate measurements were obtained during the logarithmic phase of  
30 yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean  $\pm$  SEM and were plotted against agonist



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concentration. As shown in Figure 6, the yeast cells containing the NTR1s produced an agonist-dependent growth response demonstrating that both the wild type and carboxy terminally truncated NTR1s were functional. The growth response of the rNTR1 C-term  $\Delta$  containing cells was dose-dependent giving an EC<sub>50</sub> for AcNT8-13 equal to 520 nM. This value is five fold lower than observed for cells expressing the wild type NTR1 (2.1  $\mu$ M). The carboxy terminal deletion has produced a rNTR1 that responds to a lower concentration of NTR agonist improving the sensitivity of the yeast bioassay.

Thus, deletion of a portion of the carboxy terminal intracellular domain of the rat NTR1 has produced a functional GPCR with increased agonist sensitivity when expressed in yeast, suggesting that modification of this internal domain is a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

#### **Example 7. Functional Expression of a Mutated *C. elegans* Serotonin Receptor in Yeast**

Agonists of the G protein-coupled *C. elegans* serotonin receptor (Ce 5HTR) may possess substantial nematocidal activity. These observations suggest that development of a yeast-based HTS for agonists active at Ce 5HTRs may be useful in identifying lead compounds that might be developed into nematocides with a novel mode of action. Preliminary experiments indicated that the wild type Ce 5HTR was non-functional in yeast. Thus, an effort to develop a method for improving the function of the Ce 5HTR in yeast was mounted, via replacement of the Ce 5HTR IC3 with the functional IC3 from the M3 MAR IC3 $\Delta$ .

In order to construct the IC3 replacement, PCR fragments encoding three domains were prepared by standard means. Fragment 1 contains the amino terminal-coding portion of the Ce 5HTR to the intracellular interface of the 5th TMD, amplified by PCR using oligonucleotides (AAAAGATCTAAAATGATCGACGAGACGCTTC) (SEQ ID NO: 27) and (CCGCTTGGTGATCTGACTTCTGGTTTCTGTCCCAGAGGCCTGTAGGCCAGCCAGCTCTTTGGTACGCTTCTCAGTTTCCTTATAGATCTTCCAGTACACGC

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AAATTATTGC) (SEQ ID NO: 28). The second fragment of the Ce 5HTR contains carboxy-terminal coding sequences starting proximal to the intracellular interface of the 6th TMD, amplified by PCR using oligonucleotides (AAAACTCGAGTCAATAATCGTGAATAAGGCA) (SEQ ID NO: 29) and (GGCCTACAGGCCTCTGGGACAGAAACCAGAAGTCAGATCACCAAGCGGA AGAGGATGTCGCTCATCAAGGAGAAGAAGGCCGCCAGAACGCTAGCAAT TATTACAGGTAC) (SEQ ID NO: 30). The M3 MAR IC3Δ domain was amplified by PCR as described in Example 1.

These fragments were isolated, mixed and amplified by PCR with 5' (AAAAGATCTAAAATGATCGACGAGACGCTTC) (SEQ ID NO: 27) and 3' (AAAACTCGAGTCAATAATCGTGAATAAGGCA) (SEQ ID NO: 29) oligonucleotides. The resulting fragment was digested with appropriate restriction endonucleases and assembled in p426GPD to place the mutated Ce 5HTR under control of the GPD promoter. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al.* 1998.

Yeast cells containing the Ce 5HTR were assayed in liquid culture. The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 2 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (200 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 2.0 μl of serially-diluted samples (10<sup>-2</sup> -10<sup>-9</sup> M) of serotonin (5HT). In similar reactions, the serotonergic antagonists lisuride and mianserin were added to each well at 10 μM. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements were obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM, plotted vs. agonist concentration. Ki values were

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determined using the equation of Cheng and Pruesoff. (*Y. Cheng et al. (1973)*). As shown in Figure 7, the yeast cells containing the mutated Ce 5HTR containing the M3 MAR IC3 $\Delta$  produced an agonist-dependent growth response, demonstrating that the Ce 5HTR-M3 MAR IC3 $\Delta$  is functional. As expected, the serotonergic antagonists lisuride and mianserin blocked the growth inducing effect of serotonin, demonstrating that the Ce 5HTR exhibits the expected pharmacological properties when expressed in yeast.

Thus, replacing IC3 of the Ce 5HTR with the functional deleted IC3 from the rat M3 MAR produces a functional chimeric GPCR when expressed in yeast, indicating that this method of replacing internal domains may be a generalizable method for improving the functional expression of heterologous GPCRs in cell-based assays, such as yeast assays.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

The references cited herein are specifically incorporated by reference in their entirety.

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**J. Wess**, "G-Protein-Coupled Receptors: Molecular Mechanisms Involved in Receptor Activation and Selectivity of G-Protein Recognition," *FASEB J.*, 11: 346-354 (1997).

- 24 -

We claim:

1. An isolated nucleic acid sequence having a sequence encoding a modified G protein-coupled receptor (GPCR), wherein the modification comprises a mutation in an intracellular domain of the G protein-coupled receptor and results in an improved functional response in a cell-based assay, and wherein the modified G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, a somatostatin receptor, an alpha 2A adrenergic receptor, and a serotonin receptor.
2. The nucleic acid sequence according to claim 1, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist.
3. The nucleic acid according to claim 2, wherein the modification results in improved coupling between the receptor and a heterotrimeric G protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.
4. The nucleic acid according to claim 1, wherein the mutation is a deletion.
5. The nucleic acid according to claim 4, wherein the deletion is a point mutation.
6. The nucleic acid according to claim 4, wherein the deletion is in the third intracellular loop of the G protein-coupled receptor.
7. The nucleic acid according to claim 6, wherein the G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, and an alpha 2A adrenergic receptor.
8. The nucleic acid sequence according to claim 1, wherein the serotonin receptor is Ce 5HTR.
9. The nucleic acid sequence according to claim 1, wherein the muscarinic acetylcholine receptor is a rat M3 muscarinic acetylcholine receptor or a *D. melanogaster* muscarinic acetylcholine receptor.
10. The nucleic acid sequence according to claim 1, wherein the cholecystokinin CCKB receptor is a rat cholecystokinin CCKB receptor.

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11. The nucleic acid sequence according to claim 1, wherein the somatostatin receptor is a rat somatostatin receptor subtype 3.

12. The nucleic acid sequence according to claim 1, wherein the alpha 2A adrenergic receptor is a human alpha 2A adrenergic receptor.

13. A modified G protein-coupled receptor encoded by the nucleic acid sequence according to claim 1.

14. The G protein-coupled receptor according to claim 13, wherein the deleted third intracellular loop is 44 amino acids in length.

15. A mutant G protein-coupled receptor encoded by the nucleic acid sequence according to claim 10, wherein a sequence Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO:15) is deleted from the third intracellular loop of the mutant G protein-coupled receptor.

16. An isolated nucleic acid having a sequence encoding a chimeric G protein-coupled receptor, wherein the chimeric G protein-coupled receptor comprises a modified intracellular domain of a G protein-coupled receptor that confers an improved functional response to the chimeric G protein-coupled receptor in a cell-based assay.

17. The nucleic acid according to claim 16, wherein the modified intracellular domain is the third intracellular loop.

18. A vector comprising the nucleic acid sequence according to claim 1 or 16.

19. A host cell transformed with a vector according to claim 10.

20. The host cell according to claim 19, further comprising a plasmid comprising an inducible reporter gene.

21. The host cell according to claim 19, wherein the host cell is a eukaryotic cell.

22. The host cell according to claim 21, wherein the eukaryotic cell is a yeast cell.

23. The host cell according to claim 22, further comprising a plasmid comprising an inducible reporter gene.

24. The host cell according to claim 23, wherein the reporter gene is a green fluorescent protein.

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25. The host cell according to claim 24, wherein the green fluorescent protein is operably linked to a *FUS2* promoter.

26. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the host cell according to claim 19 to a test compound; and
- (b) measuring the effect of the test compound on cell growth.

27. A host cell comprising a heterologous G protein-coupled receptor, wherein the G protein-coupled receptor has a modification that results in an improved functional response of the G protein-coupled receptor in a cell-based assay.

28. The host cell according to claim 27, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist

29. The host cell according to claim 28, wherein the modification results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the heterologous G protein-coupled receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.

30. The host cell according to claim 27, wherein the host cell is a eukaryotic cell.

31. The host cell according to claim 30, wherein the heterologous G protein-coupled receptor is modified at an intracellular domain.

32. The host cell according to claim 31, wherein the intracellular domain is the third intracellular domain.

33. The host cell according to claim 27, wherein the heterologous G protein-coupled receptor is modified at the carboxy terminal tail of the G protein-coupled receptor.

34. The host cell according to claim 31 or 33, wherein the host cell is yeast.

35. The host cell according to claim 34, wherein the modified G protein-coupled receptor is a neurotensin receptor.

36. The host cell according to claim 35, wherein the neurotensin receptor is a rat neurotensin NT1 receptor.



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37. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- (a) subjecting the host cell according to claim 27 to a test compound; and
- (b) measuring the effect of the test compound on yeast cell growth.

## SEQUENCE LISTING

<110> Pausch, Mark Henry  
Wess, Jurgen

<120> METHODS FOR IMPROVING THE FUNCTION OF HETERLOGOUS G  
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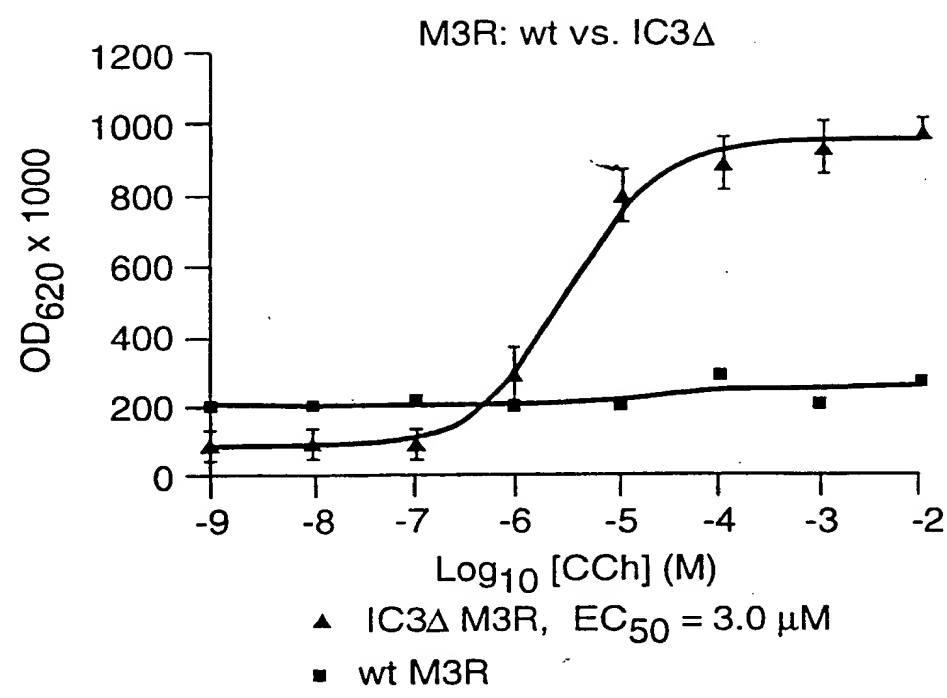
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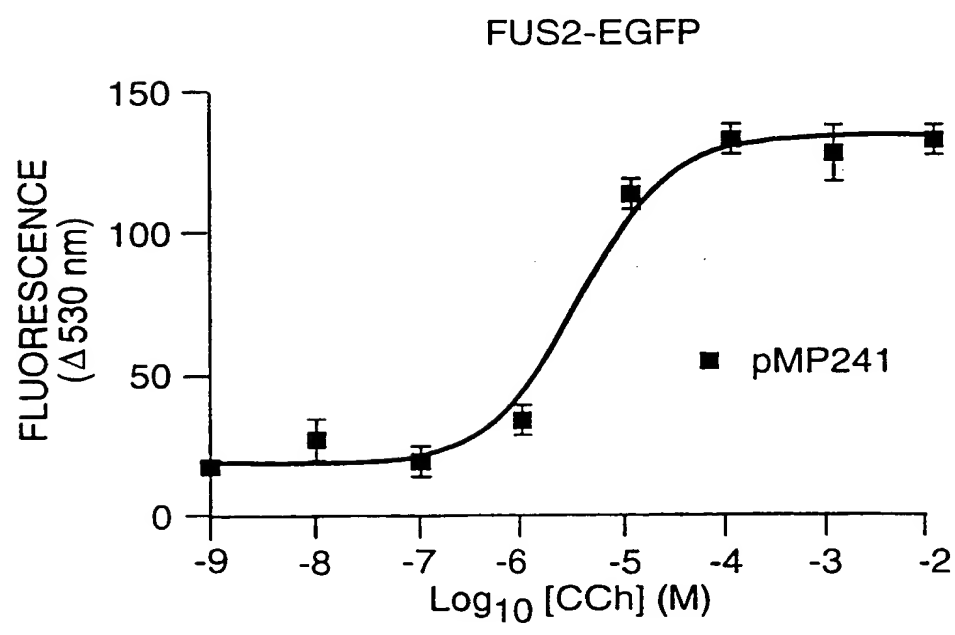
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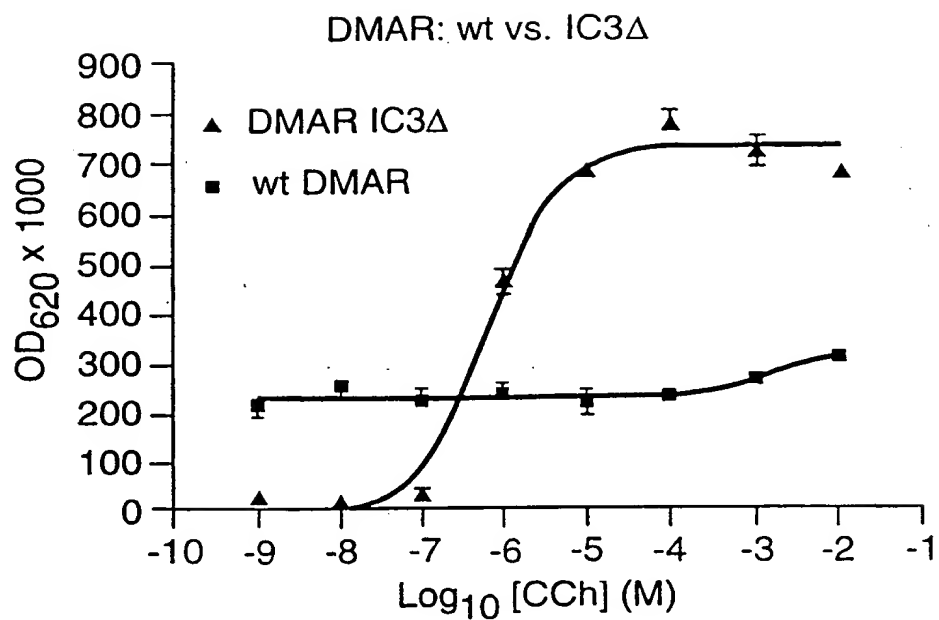
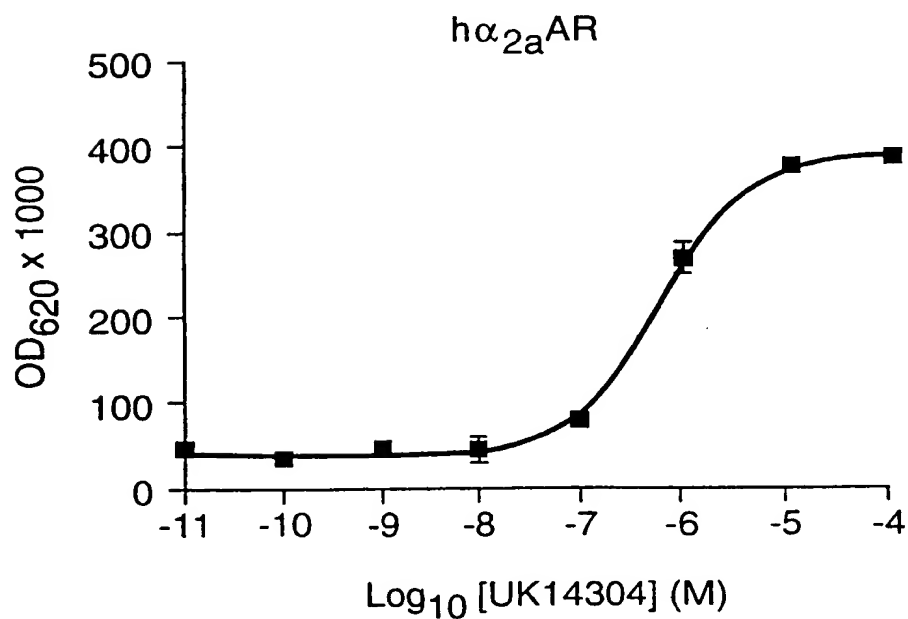


**FIG. 1A**

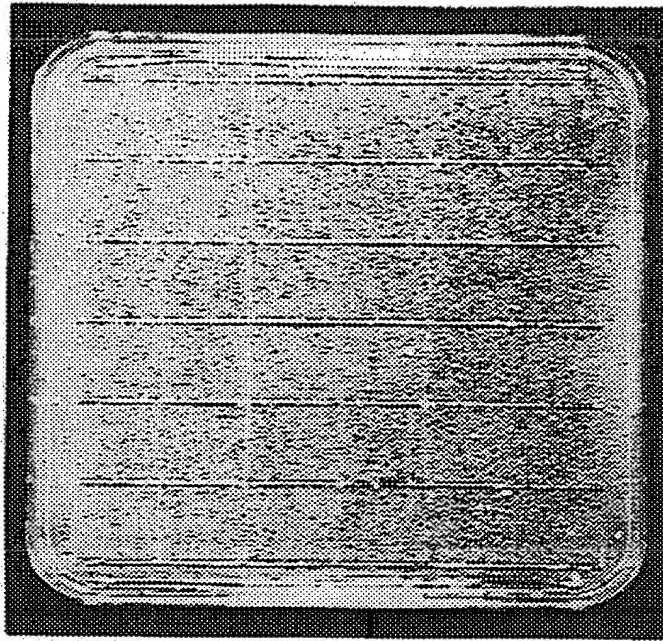
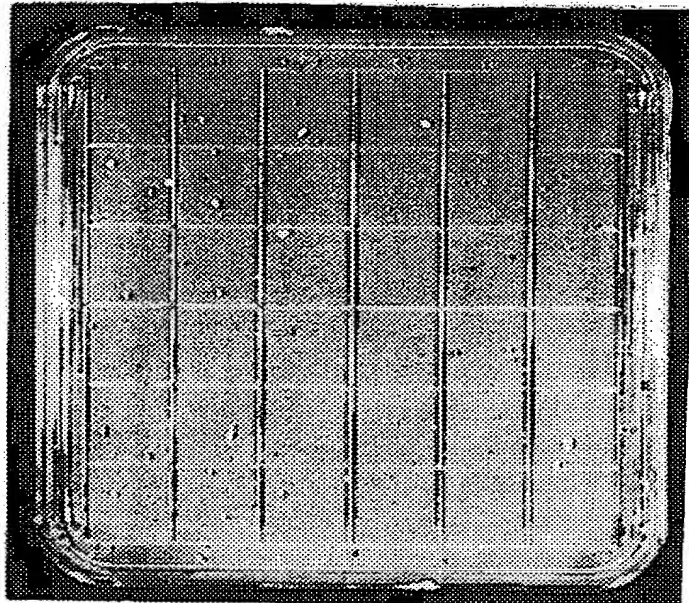


**FIG. 1B**

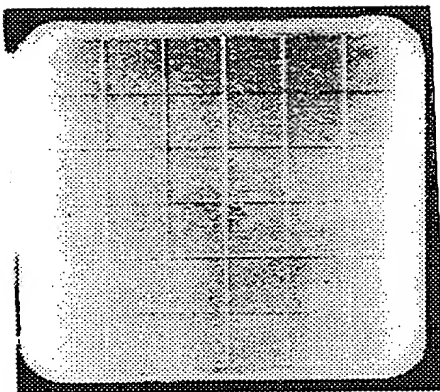
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**FIG. 2****FIG. 5**

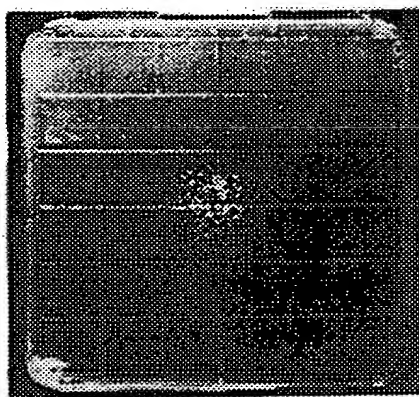
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*FIG. 3A**FIG. 3B*

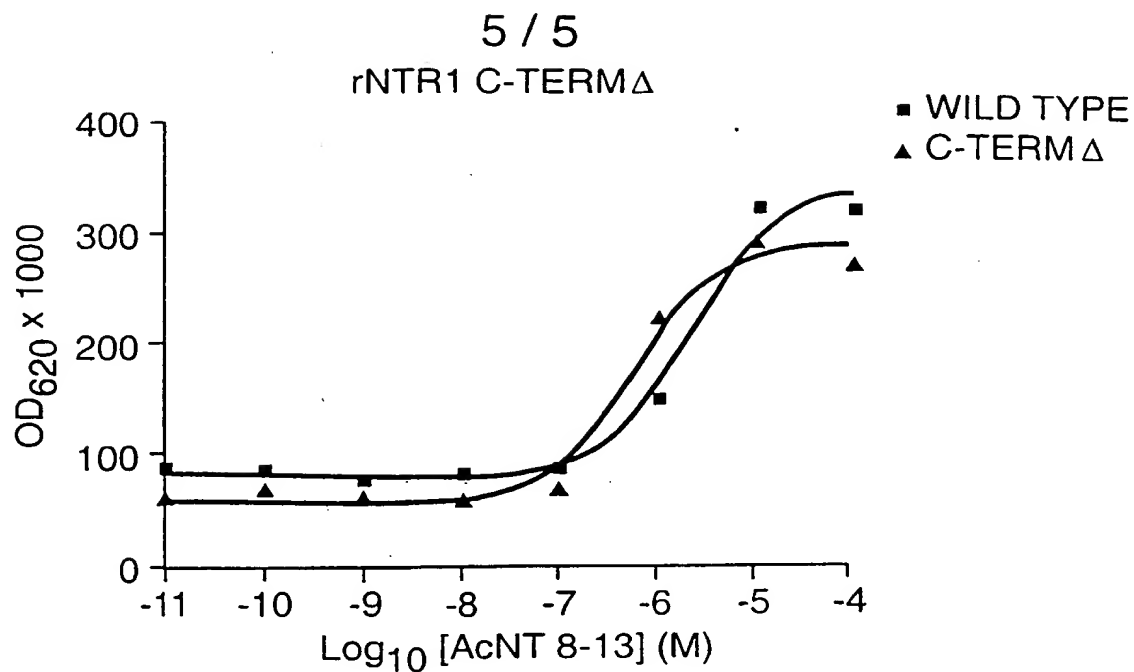
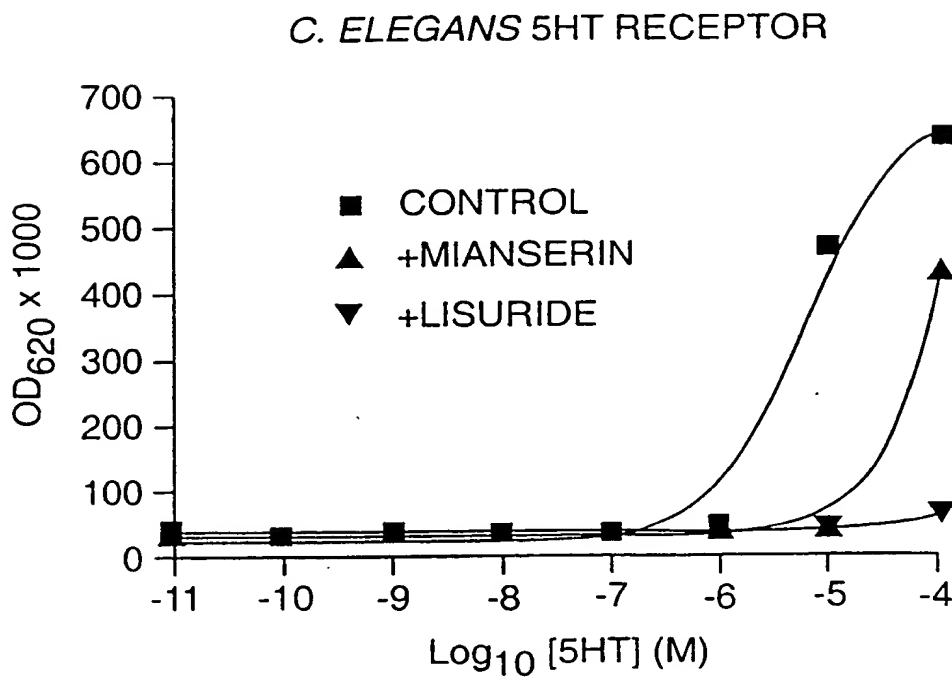
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*FIG. 4A*



*FIG. 4B*

**FIG. 6****FIG. 7**

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JC02 Rec'd PCT/PTO 01 MAR 2001

# ANNEXES

09/786033

Rec'd PCT/PTO 01 MAR 2001

We claim:

1. A yeast cell comprising a nucleic acid sequence encoding a modified, heterologous G protein-coupled receptor (GPCR), wherein the modification comprises a mutation in an intracellular domain of the G protein-coupled receptor and results in an improved functional response in a cell-based assay as compared to a wild-type form of the heterologous G protein-coupled receptor, and wherein the modified G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, a somatostatin receptor, an alpha 2A adrenergic receptor, and a serotonin receptor.
2. The yeast cell according to claim 1, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist.
3. The yeast cell according to claim 2, wherein the modification results in improved coupling between the receptor and a heterotrimeric G protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.
4. The yeast cell according to claim 1, wherein the mutation is a deletion.
5. The yeast cell according to claim 4, wherein the deletion is a point mutation.
6. The yeast cell according to claim 4, wherein the deletion is in the third intracellular loop of the G protein-coupled receptor.
7. The yeast cell according to claim 6, wherein the G protein-coupled receptor is selected from the group consisting of a muscarinic acetylcholine receptor, a cholecystokinin CCKB receptor, and an alpha 2A adrenergic receptor.
8. The yeast cell according to claim 1, wherein the serotonin receptor is Ce 5HTR.
9. The yeast cell according to claim 1, wherein the muscarinic acetylcholine receptor is a rat M3 muscarinic acetylcholine receptor or a *D. melanogaster* muscarinic acetylcholine receptor.
10. The yeast cell according to claim 1, wherein the cholecystokinin CCKB receptor is a rat cholecystokinin CCKB receptor.
11. The yeast cell according to claim 1, wherein the somatostatin receptor is a rat somatostatin receptor subtype 3.

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12. The yeast cell according to claim 1, wherein the alpha 2A adrenergic receptor is a human alpha 2A adrenergic receptor.

13 14. The yeast cell according to claim 6, wherein the deleted third intracellular loop is 44 amino acids in length.

1.126

14 15. The yeast cell according to claim 11, wherein a sequence Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO:15) is deleted from the third intracellular loop of the mutant G protein-coupled receptor.

15 16. A yeast cell comprising a nucleic acid sequence encoding a chimeric G protein-coupled receptor, wherein the chimeric G protein-coupled receptor comprises a first heterologous G protein-coupled receptor in which an intracellular domain has been replaced with a modified intracellular domain of a second heterologous G protein-coupled receptor, and wherein the modified intracellular domain of the second heterologous G protein-coupled receptor confers an improved functional response to the chimeric G protein-coupled receptor in a cell-based assay as compared to a wild-type form of the first heterologous G protein-coupled receptor.

16 17. The yeast cell according to claim <sup>15</sup>16, wherein the modified intracellular domain is the third intracellular loop.

17 20. The yeast cell according to claim 1 or <sup>15</sup>16, further comprising a plasmid comprising an inducible reporter gene.

18 24. The yeast cell according to claim <sup>17</sup>20, wherein the reporter gene is a green fluorescent protein.

19 25. The yeast cell according to claim <sup>18</sup>24, wherein the green fluorescent protein is operably linked to a *FUS2* promoter.

20 26. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- <sup>15</sup>
- (a) subjecting the yeast cell according to claim 1 or <sup>15</sup>16 to a test compound; and
  - (b) measuring the effect of the test compound on cell growth.

21 27. A yeast cell comprising a heterologous G protein-coupled receptor, wherein the G protein-coupled receptor has a deletion in an intracellular domain that results in an improved functional response of the G protein-coupled receptor in a cell-based assay as compared to a wild-type form of the heterologous G protein-coupled receptor.



<sup>21</sup>  
22 -28. The yeast cell according to claim <sup>22</sup>27, wherein the modification promotes agonist stimulated growth, and wherein the agonist is a G protein-coupled receptor agonist

<sup>22</sup>  
23 -29. The yeast cell according to claim <sup>21</sup>28, wherein the modification results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the heterologous G protein-coupled receptor to interact with cell desensitization or sequestration-internalization machinery or proper plasma membrane localization.

<sup>21</sup>  
24 -32. The yeast cell according to claim <sup>21</sup>27, wherein the intracellular domain is the third intracellular domain.

<sup>21</sup>  
25 -33. The yeast cell according to claim <sup>25</sup>27, wherein the heterologous G protein-coupled receptor is modified at the carboxy terminal tail of the G protein-coupled receptor.

<sup>25</sup>  
26 -35. The yeast cell according to claim <sup>26</sup>33, wherein the modified G protein-coupled receptor is a neurotensin receptor.

<sup>26</sup>  
27 -36. The yeast cell according to claim <sup>21</sup>35, wherein the neurotensin receptor is a rat neurotensin NT1 receptor.

<sup>21</sup>  
28 -37. A method for screening compounds capable of binding to G protein-coupled receptors comprising:

- <sup>21</sup>  
(a) subjecting the yeast cell according to claim <sup>21</sup>27 to a test compound; and  
(b) measuring the effect of the test compound on yeast cell growth.

<sup>21</sup>  
29 -38. The yeast cell according to claim 6, wherein the deletion is IC3A.